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Molecular Cloning & Bioinformatics characterization of ATP-Binding Cassette (ABC) Transporters from *Toxoplasma gondii*

Honours Thesis in Molecular Biology, 30 hp (MB701A)

(15/05/2008 -20/09/2008)

Report Version II

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Abstract

Title: Molecular Cloning & Expression Analysis of ATP-Binding Cassette (ABC) Transporters from *Toxoplasma gondii*

Dept: Molecular Biology, University of Skövde Sweden

Course: Honours Thesis in Molecular Biology, 30 hp (MB701A)

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Toxoplasma gondii is an obligate intracellular protozoan parasite of the subphylum Apicomplexa and causes toxoplasmosis in immunocompromised individuals and infected infants. *T.gondii* is uniquely adapted to the intracellular parasitism, being able to invade and survive in a wide range of cell types. Successful replication of the parasite within its parasitophorous vacuole necessitates substantial membrane biogenesis. The parasite meets its great demand of phospholipids by *de novo* phospholipids synthesis or by scavenging pre synthesized complex lipids and their precursors from the host cell.

T. gondii has 14 chromosomes and 8032 annotated genes currently available in Toxo DB. ATP-Binding Cassette (ABC) transporters represent the largest evolutionarily conserved super family of proteins involved in lipid and drug transport.

The aim of this project was to determine the classification and domain organization of TgABC transporters by using bioinformatics tools and molecular cloning of ABC transporters from *T. gondii* in to yeast vector for their expression in *S.Cerevisiae* (AD-18) mutant strain. For this purpose, six TgABC transporters genes were screen out and classification was done on the basis of their size, exon number, chromosomal location, domain organization and assigned families and subfamilies by amino acid sequence similarity to ABC proteins of other organisms.

Key words: *Toxoplasma gondii*, ATP binding cassette transporter, Resistance, Phospholipids synthesis

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Abbreviations

ABC	ATP-Binding Cassette
Tg-ABC	<i>Toxoplasma gondii</i> ATP-Binding Cassette
ABC G2	ABC superfamily G member 2
AIDS	Acquired immunodeficiency syndrome
NBD	Nucleotide-binding domain
TMD	Transmembrane domain
PCR	Polymearse Chain Reaction
S.Cerevisiae	Saccharomyces cerevisiae
HIV	Human immunodeficiency virus
GAL	UDP- glucose-4- epimerase
LB	Laria Bartani
DMSO	Dimethlsulfoxid
E. Coli	Escherichia coli
YPD	Yeast peptone dextrose
SD	Synthetic dextrose

1 Introduction

The word Toxoplasma (*toxos* = arc, *plasma* = form) is derived from its crescent shape. *Toxoplasma gondii* is an intracellular protozoan parasite (Smith 1995) which infects humans as well as wide variety of mammals and birds (Hill *et al.*, 1998). The organism was first discovered by Nicolle and Manceaux (1908) as a tissue parasite of *gondii* (an African rodent) and Darling found it in Man (Subash 1990). The infection has been confirmed in 200 species of mammals including man and in Domestic / Wild felines, which serve as a definitive host (Pedro *et al.*, 2003).

Toxoplasmosis is found throughout the world (Dubey 1999). *T. gondii* can cause severe acquired infection in animals and human being, which may be localized or generalized. Lymphadenitis is the most frequently observed clinical sign (deep cervical nodes). Encephalitis is an important sign of Toxoplasma in later stages. During the 1980,s Toxoplasmic encephalitis in human emerged as a common complication associated with AIDS (Subash, 1990). Congenital infection in animals and pregnant women develop the most serious side effects leading to spontaneous abortion, still birth, birth defects, mummification, neonatal losses or fetal abnormalities. The mechanism of vertical transmission is not yet understood (Remington *et al.*, 1995).

The treatment and prophylaxis of *T. gondii* infection is one of the most serious problem facing all over the world. Approximately 500 million populations are estimated to have antibodies to *T. gondii* infection (Subash Ch.1990). The Studies show that 16% to 40% of the human populations in North America and Great Britain, 50% to 80% of the populations in Europe and Latin America have antibodies against *T. gondii*, indicating that they harbor infection at some time (Pedro *et al.*, 2003).

T. gondii exhibits a predator-prey type life cycle having two phases (Torada 2001). The sexual phase of the infection occurs only in felines when the cysts are ingested by a cat and the parasite multiplies asexually by merogony and then sexually by gametogony to produce oocyst (Pedro *et al.*, 2003). The asexual phase occurs in the intermediate host, when sporulated oocyst penetrates through intestinal wall and spread by haematogenous route. This stage is called tachyzoite that infect tissues and replicate intracellularly. As a result there is formation of tissue cysts containing bradyzoites. (Urquhart *et al.*, 1996).

The source of transmission is the ingestion of vegetables, fruits, water, soil, food contaminated by cat faces, raw or undercooked meat. Flies and Cockroaches may act as a mechanical carrier to transfer oocysts to different variety of foods. Other sources include transplacental transmission, from mother to the offspring through milk, transplantation of organs, transfusion of blood and venereal transmission (Pedro *et al.*, 2003).

Tachyzoites (2 X 7 μ m) contain a unique cytoskeleton (subpellicular microtubules, conoid), secretory organelles (rhoptries (6-12), micronemes (50-100), dense granules) along with apical complex, endosymbiotic organelles (mitochondria, apicoplast), eukaryotic organelles (nucleus, endoplasmic reticulum, golgi bodies, ribosomes) and specific structures (acidocalcisomes) (Louis 2007). Secretory organelles play an important role in the host cell invasion and maturation of the parasitophorous vacuole.

Dense granules release their contents after invasion has completed, rhoptries release their contents as invasion proceeds and micronemes release their contents during attachment process in parasitophorous vacuole (Black & Boothroyd 2001). The Conoid are thimble shape structure at the extreme apex of the parasite. It is connected with two apical and polar rings. Two microtubules extend from apical ring and terminate within the body of the cell. Polar ring function as a microtubule organization center from which 22 subpellicular microtubules move down toward the parasite body. Apicoplast are responsible for the synthesis of fatty acids, lipoic acid and isoprenoid precursors (Louis 2007).

More than hundred ABC transporters have been described, and each one is specific for a particular substrate including lipids, sugars, peptides, inorganic ions, cell surface components, iron, sulfur, heavy metals and drugs. Although most family members have been described in prokaryotes, the number is rapidly increasing in eukaryotes. The physiological roles of ABC transporters range from transporting nutrients (in prokaryotes) and the efflux of waste products to regulation. Usually they are low capacity, but high affinity transporters, able to transport substrates against the concentration gradient up to more than 10 000 fold. Characteristically ABC transporters are comparatively specific for a particular set of substrates (Alberts. *et al.*, 2002).

The ABC transporter proteins represent the largest super family found in most organisms including bacteria, yeast, plants and protozoa. The members of ABC transporter super family contain a highly conserved ATP-binding cassette, whose amino acid sequence display three major conserved motifs (Walker A & B, ABC signature). ABC transporter has two types of structural/domain organization. Full size ABC proteins consist of four structural domains having two cytoplasmic domains called ATP or nucleotide-binding domain (NBD) and two transmembrane domains (TMD). A number of ABC proteins are half size transporter consisting of single NBD and TMD (Virginie. *et al.*, 2006).

T. gondii has fourteen chromosomes and 8032 annotated genes currently available in Toxo data base (2008). The aim of this project was to determine the classification and domain organization of TgABC transporters by using bioinformatics tools and cloning of ABC Transporters from *T. gondii* in to yeast vector for their expression in *S.Cerevisiae* (AD-18) mutant strain. It was predicated that ABC transporters of *T. gondii* may function as a multi drug efflux pump and lipid transport. For this purpose, six TgABC transporters genes were screen out and classification was done on the basis of their size, exon number, chromosomal location, domain organization (toxos data base, 2008) and assigned families and subfamilies by amino acid sequence similarity to ABC proteins of other organisms using BLAST program (transport classification data base, 2008).

2 Materials and Methods

2.1. Annotation of *T.gondii* ABC Transporters:

Six genes encoding putative ABC transporters of *T.gondii* were identified and screen out from *T.gondii* database (2008). Identification of sorting signal was performed by using the program signalP (2008). In addition human and protozoan ABC sequences were used as queries for Blast searches (transport classification data base, 2008).

2.2. Gene purification from pCDNA 3.1 + Vector:

Six ABC transporters genes were retrieved from pCDNA 3.1 + Vector and simply (Appendix I). The plasmid was absorbed on filter paper, which was cut out to recover the DNA. Tris-HCl was added and filter was hydrate for 5 minutes. After a brief centrifugation supernatant was used to transform in XL-1b competent cells (Stratagene USA) according the laboratory compendium (Sazzad & Magnus, 2008). Purification was done from the culture of bacteria harbouring pCDNA 3.1 + plasmid along with TgABC clones by using Miniprep kit (Analytik Jean Germany).

2.3. Polymearse Chain Reaction (PCR) Amlification:

The pCDNA 3.1 + clones containing TgABC cDNA were used as a templete for gene specific PCR reactions. Master Mix was prepared for PCR having all the reagents and its composition is given in Phusion high fidelity PCR kit (Stratagene USA). The thermocycler was run according to the program given in Phusion high fidelity PCR kit. Optimization of annealing temperature (T_A) was varied between 50°C to 65°C for individual PCR. Gene specific primers (Invitrogen Germany) were designed contain *NotI* restriction site, maximum length of the primers varied between 31 to 32, melting temperature varied between 59°C to 69°C and guanine and cytosine content was 70% to 75% (Primer3: Pick primers from a DNA sequence, 2008) was given in Table 1.

Table1: Description of TgABC Genes Primers (Forward and Reverse).

Assigned Name	Forward Primer	Reverse Primer	TA	TM	Length	GC %
TgABC-A	CTCGCGGCCGC	CTCGCGGCCGCCT	61 ° C	69 ° C	FR 31	FR 74
	ATGGACGTCGG	ACCCACGCCCGCG			RP 29	RP 82
	GCCTCGAGT	AGC				
TgABC-B	CTCGCGGCCGC	CTCGCGGCCGCTC	57 ° C	60 ° C	FR 30	FR 63
	ATGCCCTCGCA	AGGACTTCAGTTTA			RP 38	RP 50
	TCCTAATT	AGAGATTTCTT				
TgABC-C	CTCGCGGCCGC	CTCGCGGCCGCTC	57 ° C	63 ° C	FR 31	FR 67
	ATGGACTTTCTG	ACGCTTCAAGGCC			RP 29	RP 68
	CGCAGACG	GAT				

TgABC-D	CTCGCGGCCGC ATGGCAGACTC TGCCCTC	CTCGCGGCCGCTT AGTTTCGGCGATT AAGAT	58° C	59° C	FR 29 RP 32	FR 72 RP 56
TgABC-E	CTCGCGGCCG CATGAATTCC CTCGCTGGC	CTCGCGGCCGCT CATTGGTTCTCCT TCGTTTCG	57° C	61°	FR 29 RP 32	FR 68 RP 62
TgABC-F	CTCGCGGCCG CATGGACGCT CCGGACATGA	CTCGCGGCCGCT CAGCGACGAAGC CGGAC	62° C	66° C	FR 31 RP 29	FR 70 RP 75

NotI is the Restriction site of all above primers.

The 1% agarose gel (Carl Roth Germany) was prepared according to the instruction given in the laboratory compendium (Karim and Magnus, 2008) Run the gel at 100-120 mA for 1-2 hours. The separation of DNA fragments was analyzed by UV irradiation of the gel. The PCR product was purified by pure-link PCR purification kit (Invitrogen Germany) and concentration was measured by the Nanodrop method.

2.4. Digestion of ABC transporters genes:

The digestion was performed by *NotI* restriction enzyme (Invitrogen Germany) at 37° C incubation for 2 hour. After digestion, DNA was cleaned with pure-link PCR purification kit (Invitrogen Germany) and the concentration was measured by the Nanodrop method.

2.5. Purification of pESC-Ura Plasmid:

The purification of plasmid was performed from overnight culture of bacteria harbouring plasmid pESC-Ura (Appendix IV). The protocol was given in the plasmid miniprep kit (Invitrogen Germany). Concentration of purified vector was measured by the Nanodrop method.

2.6. Digestion of pESC-Ura plasmid DNA and gel electrophoresis:

Plasmid DNA was digested with *NotI* restriction enzyme at 37° C incubation for 2 hours. Digested plasmid DNA was loaded in the 1% agarose gel along with ladder and visualized under ultraviolet irradiation. The fragment of digested product was cut out from gel and cleaned with gel extraction kit (Invitrogen Germany). The concentration was measured by the Nanodrop method. For symmetric (non directional) cloning, the linearized vector was dephosphorylated and purified. Finally the concentration of dephosphorylated plasmid DNA was measured by Nanodrop method.

2.7. Ligation of digested vector (pESC-Ura) and insert:

NotI digested pESC-Ura and insert DNA were used for ligation at molar ratio of 1:3 and 1:5 at 16° C for 12 hrs. The use of purified water instead of the insert served as a negative control to determine the re-ligation efficiency of empty vector.

2.8. Preparation and Transformation of E. Coli XL-1b competent cells:

L.B. media was inoculated with XL-1b cells and incubated overnight at 37° C. The further procedure was done according to the protocol of Stratagene USA. XL-1b competent cells were mixed with ligation mixture. The cells were plated on L.B plate with antibiotics ampicillin at 37°C for 16 hours. All the procedures were done according to the according the laboratory compendium (Sazzad & Magnus 2008).

2.9. Colony PCR for verification of vector with insert:

Colony PCR consists of three steps of denaturation, annealing, extension and total PCR cycles were 30 that was run according to the program given in Dream Taq PCR kit (Fermentas Germany). Individual colonies were examined to verify the presence of inserts containing pESC-URA plasmid by colony PCR. The colony was picked up from the plate and resuspended in HPLC-grade water (Carl Roth Germany). The suspension was used as a template for colony PCR using gene specific primers (Table 1) and flanking vector primers (Table 2). Master Mix was prepared containing Dream Taq PCR kit (Fermentas Germany).

Table 2: Description of pESC –Ura flank specific primer (Forward and Reverse).

pESC-Ura	Forward Primer Sequence (5-3)	Reverse Primer Sequence (5-3)
GAL10	GGTGGTAATCCATGTAATATG	GGCAAGGTAGACAAGCCGACAAC

The PCR products were analysed for the size of gene inserted in to the vector on an agarose gel. The forward and reverse PCR primers flank the multiple cloning sites. Positive bacterial clones with insert were grown overnight in LB media at 37 °C. Purification was done from the culture of bacteria harbouring pESC-Ura plasmid along with TgABC clones by using Miniprep kit (Analytik Jean Germany).

2.10. Restriction digestion for verification of insert orientation:

pESC-Ura clones containing cDNA was digested at 37° C for 2 hours. Restriction summaries of both genes (New England biolab international, 2008) and vector (Stratagene an agilent technologies division, 2008) are given in Appendix II & III. Digested product was loaded in the agarose gel along with DNA ladder. The product fragments were visualized by ultraviolet irradiation for verification.

2.11. Sequencing of cloned genes:

TgABC-B, TgABC-D and TgABC-F in pESC-Ura were sequenced by Agowa GmbH using GAL10-Fwd and GAL10 Rev Primers.

2.12. Preparation and Expression of Yeast competent cells (*S. cerevisiae*):

S. cerevisiae AD-18 lacking ABC transporters (Yeast genetic resource center, 2008) was taken from the frozen glycerol stock and streaked on YPD media. The further procedure was done according to the protocol of Stratagene USA. Yeast expression plasmid was added with salmon sperm DNA (Invitrogen Germany). All other procedures were done according to the laboratory compendium (Nishith 2008).

3 Results and Discussion:

Toxoplasmosis is one of the most common infections in men and animals cause by protozoan parasite *T. gondii*, which is responsible for significantly higher morbidity and mortality in both human and warm-blooded animals. Toxoplasmosis is world wide distribution, zoonotic in nature and depending upon the geographic location. 15-85% of the global population can be symptomatically infected (Subash 1990). Toxoplasmosis is also responsible for abortion and congenital defects in human and domestic livestock including sheep, goat, camel, cow and buffaloe (Pedro *et. al.*, 2003).

ABC transporters were first discovered and studied in bacteria in the fifties but in 1976, 170 kDa glycoprotein was identified in the plasma membrane of multidrug resistance cells (MDR) and termed P-glycoprotein. In protozoans, the first Pgp-like ABC transporter was described in the human malaria parasite *P. falciparum* (Pgh1 encoding by Pfmdr1). (Aline *at al.*, 2009).

ABC transporters represent the largest evolutionarily conserved super family of proteins. Virginie and his team had been identified 24 ABC transporters of *T. gondii* and their amino acid sequences exhibit all the distinctive biochemical features of the ABC family members. Fifteen of the ABC transporters of *T. gondii* cluster in to five out of seven Human ABC transporters families; six belongs to ABCB (lipid export, drug and peptide), two to ABCC (drug export and organic anion conjugate), one to each ABC-E (Rnase L inhibitor, translation regulation and antibiotics resistance) and ABCF (regulation of gene expression and drug resistance) and five to ABCG (drug export and resistance). The remaining nine ABC transporters of *T. gondii* included four from ABC-H (energy generating subunit), four from SMC (Structural maintenance of chromosomes) and one member of unclear (Virginie *et. al.*, 2006).

There are two basic aim of our study. Firstly we determined the bioinformatics classification and domain organization of five ABCG and one ABCH family of *T. gondii*

by using bioinformatics tools. Secondly molecular cloning and expression analysis of TgABC transporters genes in Yeast expression vector to find out their role in lipid and drug transport. For this purpose, *T. gondii* ABC transporters genes were cloned in pESC-Ura and expressed in *S. Cerevisia*. These findings are in concomitant with the results of Schmid *et al.*, 2009.

3.1. Classification of *T.gondii* ABC transporters:

A detailed inventory of five ABCG and one ABCH family of *T. gondii* including assigned name, chromosomal localization, sizes, overall structural organization and exons number (*T.gondii* genome data base, 2008) was presented in Table 3.

The exon encoded proteins of ABCG family of *T. gondii* are half transporters that exhibit a unique structural organization having single N-terminal ATP or nucleotide binding domain fused to a single C-terminal set of tranmembrane domain as predicted by intrpro program of *T. gondii* data base (2008) shown in Table 3. it was reported that ABCH family includes members containing only a single ABC domain (no TMD). These ABC domains are often the energy-generating domains of multi component membrane-bound transporters that are either importers (uptake system) or exporters (Virginie *et. al.*, 2006). These results shows contraversion of our results of ABCH family of *T. gondii*, that have single ATP binding domain fused to the single tranmembrane domain as predicted by intrpro program as shown in Table 3 (*T. gondii* data base, 2008). It was also predicted that there is no signal peptide at N terminus of five ABCG and one ABCH family of *T. gondii* (*T. gondii* data base, 2008)).

Table 3: TgABC transporters genes are classified on the basis of assigned families, chromosomal location, size, domain organization, exon number.

Assigned Name	Gene ID	Chromosomal Localization	Size (Nucleotide)	Size (Amino acid)	Domain structural arrangement	Exon
TgABC-A	80.m02179	IX	2649	882	NBD-TMD	7
TgABC-B	583.m05692	XI	2106	701	NBD-TMD*	6
TgABC-C	50.m03178	XII	2946	981	NBD-TMD*	11
TgABC-D	80.m00083	IX	2301	766	NBD-TMD*	10
TgABC-E	80.m02273	IX	2439	812	NBD-TMD*	15
TgABC-F	49.m05729	VI	2385	794	NBD-TMD*	11

* Nucleotide-Binding Domain (NBD), Trans Membrane Domain (TMD)

The homology search against the database of five ABCG and one ABCH family of *T. gondii* were performed using BLAST algorithm assigned ABCG family (represents the second largest family of *T. gondii* having only one subfamily termed as White subfamily) and ABCH family respectively (four members and one of them was included in our study).(transport classification data base, 2008). It was suggested that the amino acid sequencing of five ABCG and one ABCH family of *T. gondii* had closest similarity with the Human ABC superfamily G member 2 (Gene ID Q9UNQ0) genes can be seen in Table 4.

Human ABCG members ABCG1, ABCG5, ABCG8 and ABCG4 have been involved in the regulation of lipid-trafficking mechanisms in macrophages, hepatocytes, intestinal mucosa cells and brain tissue respectively. Human ABCG2 also functions as a multi drug efflux pump in some cancer cells. Its high level of expression in placenta trophoblast cells also suggests a physiological role in lipidic homeostasis and cellular detoxification. ABCG2 is a plasma membrane protein with minor fraction within the intracellular membranes (Virginie. *et al.*, 2006).

Amino acid sequences of all ABC transporters of *T.gondii* showed sequence identity between (25–41%), and similarity between (44–61%), which was consistent with findings obtained for Virginie. *et al.*, 2006. The alignment score of TgABC-D was high as 228 bits and E value of TgABC-C was low as 9e-40. E value indicate that the number of hits see when searching a database of a particular size and alignment score is the sum of the scores specific for each of the aligned pairs of letters and as the value alignment score is high when the similarity will be high.

Table 4: Family and subfamily assigned was based of BLAST results with the Transport classification database.

Assigned Name	Family	Human match (accession no)	Score (Bits)	E value	Identity	Similarity
TgABC-A	ABCG(<i>white</i>) *	ABC-G2 (Q9UNQ0)	174	6e-44	25%	44%
TgABC-B	ABCH	ABC-G2 (Q9UNQ0)	183	5e-47	41%	59%
TgABC-C	ABCG(<i>white</i>) *	ABC-G2 (Q9UNQ0)	160	9e-40	38%	57%
TgABC-D	ABCG(<i>white</i>) *	ABC-G2 (Q9UNQ0)	228	3e-60	30%	50%
TgABC-E	ABCG(<i>white</i>) *	ABC-G2 (Q9UNQ0)	184	3e-47	38%	61%
TgABC-F	ABCG(<i>white</i>) *	ABC-G2 (Q9UNQ0)	186	7e-48	37%	60%

*ABC superfamily G member 2 (ABC-G2)

3.2. Cloning and Expression analysis of TgABC transporters:

TgABC transporters genes were retrieved from pcDNA 3.1+ vector and further used for gene specific PCR reactions. The optimisation of various parameters was done in PCR reaction as described by Henrik and Elie (2007). Annealing temperature was optimised in between 50°C to 65°C (Fig. 1 a, b, c) because at these optimised values higher PCR product was obtained. Generic DNA (ABC transporters genes) was digested with *NotI* restriction enzyme and further used for ligation in pESC-Ura vector.

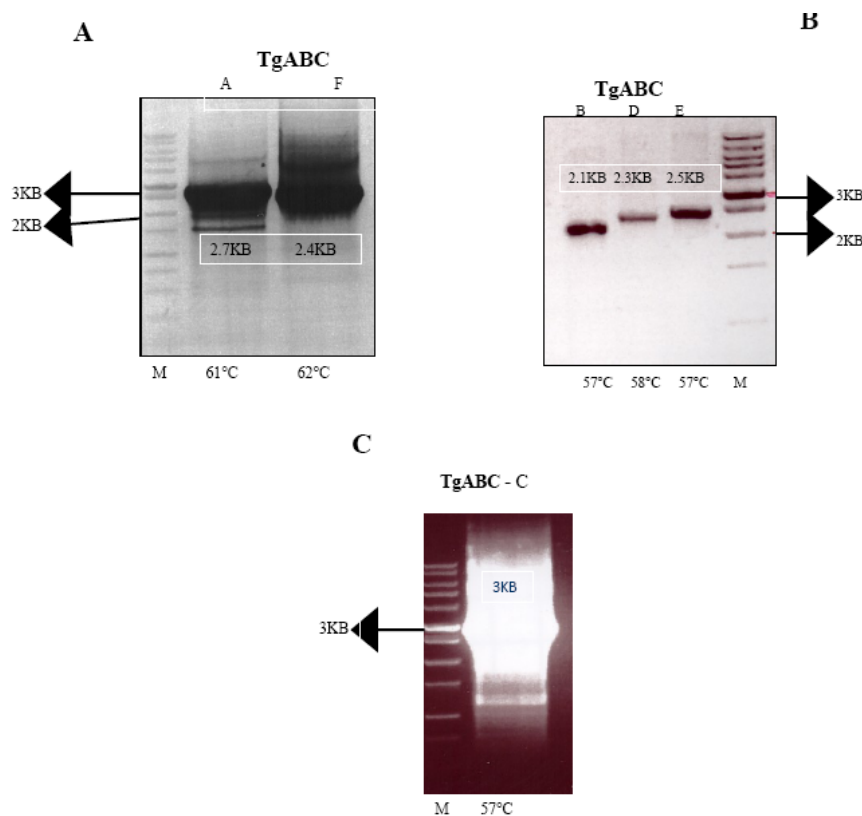


Figure 1: Amplifications with the different specific primers yielded PCR products of the expected size of TgABC. (A) Gel image show the amplification of TgABC-A & F gene product. The size of the product was 2.7 & 2.4 kb respectively and annealing temperature was optimised at 61°C. (B) Gel image show the amplification of TgABC-B, D & E gene product. The size of the product was 2.1, 2.3 & 2.5 kb respectively and annealing temperature was optimised at 57°C. (C) Gel image shows the amplification of TgABC-C gene product. The size of the product was 3kb and annealing temperature was optimised at 57°C.

pESC-Ura yeast expression was digested with *NotI* restriction enzyme along with undigested vector as a control and the degree of digestion was tested (Fig. 2). *NotI* digested pESC-Ura are cloned in to TgABC genes as described by Jung *et al.*, 2008. The use of purified water instead of insert served as a negative control to determine the relegation efficiency of empty vector. There were very few colonies seen in the LB^{AmpR} plate due to deposhorylated vector (pESC-Ura)

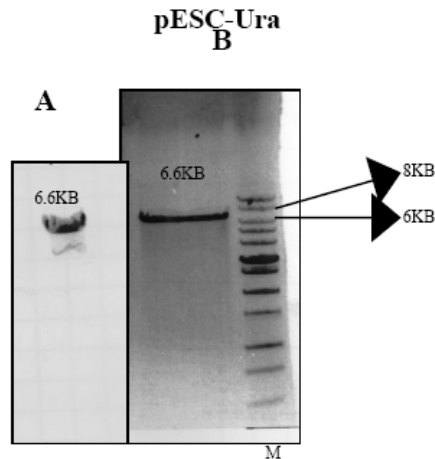
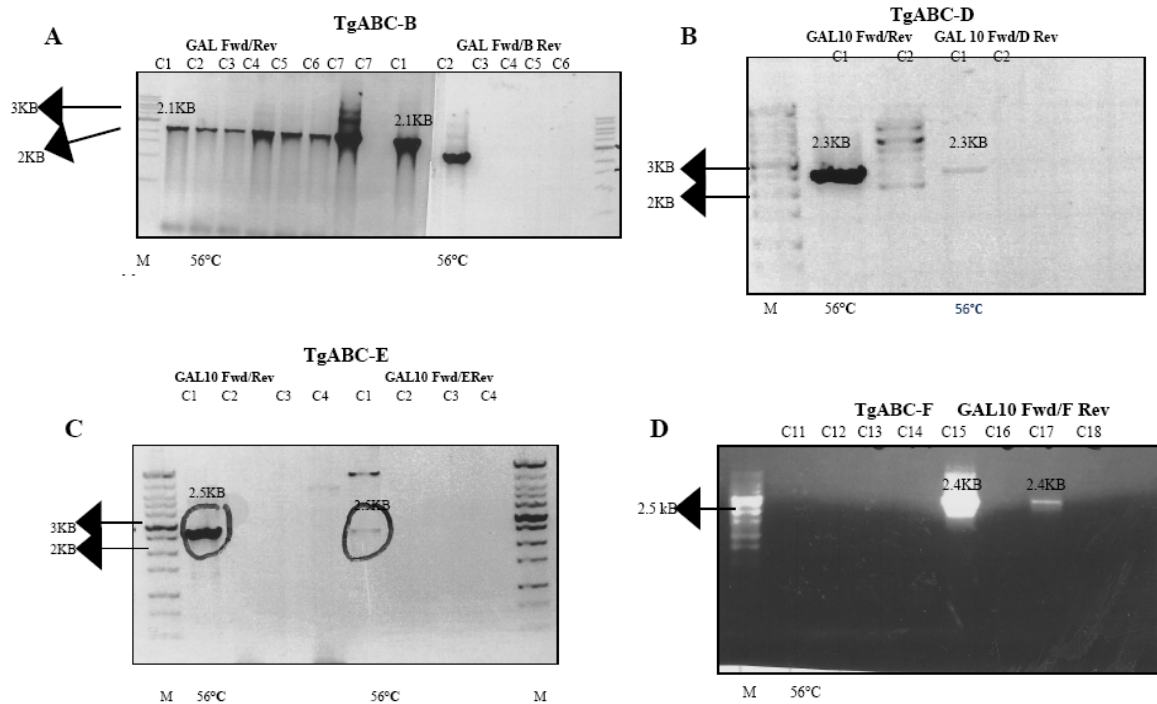


Figure 2: Fragments of pESC-Ura vector were observed. (A) Gel image show the undigested pESC-Ura vector product uses a control. The size of the product was 6.6 Kb. (B) Gel image show the digested pESC-Ura vector product. The size of the product was 6.6 Kb.

Individual colonies were examined to verify the presence of clone by PCR using gene specific primers (Table 1) or flanking vector primers (Table 2). PCR products were analysed for the size of TgABC transporters gene (Fig. 3). These findings are in concomitant with the results of Kong *et al.*, 2005. Seven bacterial colonies were found in TgABC-B LB^{AmpR} plate. TgABC-D showed only two bacterial colonies, TgABC-E revealed four colonies, TgABC-F had twenty bacterial colonies and TgABC-A had two colonies. Clone 1 & 2 of TgABC-B, clone 1 of TgABC-D, clone 1 of TgABC-E, clone 15 and 17 of TgABC-F and clone 1 of TgABC-A demonstrate the success of ligation and transformation of bacteria (Fig. 3).



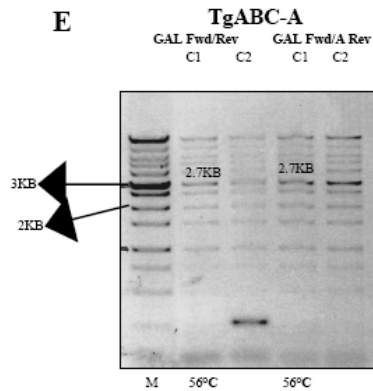


Figure 3: Amplification of different colony PCR products was shown the presence of insert in vector (pESC-URA). (A) The gel image shows the amplification of Clone 1 to 7 of TgABC-B using gene specific primers i.e. B reverse and vector specific primers i.e. GAL10 forward and reverse. Clone 1 & 2 give the positive results. (B) The gel image shows the amplification of Clone 1 & 2 of TgABC-D using gene specific primers i.e. D reverse and vector specific primers i.e. GAL10 forward and reverse. Clone 1 gives the positive results. (C) The gel image shows the amplification of Clone 1 to 4 of TgABC-E using gene specific primers i.e. E reverse and vector specific primers i.e. GAL10 forward and reverse. Clone 1 gives the positive results. (D) The gel image shows the amplification of Clone 1 to 18 of TgABC-F using gene specific primers i.e. F reverse and vector specific primers i.e. GAL10 forward and reverse. Clone 15 & 17 give the positive results. (E) The gel image shows the amplification of Clone 1 & 2 of TgABC-A using gene specific primers i.e. A reverse and vector specific primers i.e. GAL10 forward and reverse. Clone 1 gives the positive results.

Miniprep was performed for bacteria harbouring pESC-Ura vector along with insert and then digested with restriction enzymes for mapping (Fig. 4) as described by Sayada et al., 1995. To test the orientation of TgABC-A, TgABC-B, TgABC-D, TgABC-E & TgABC-F clones, diagnostic restriction mapping was performed in adding a set of restriction enzymes as shown in Table 5.

Table 5: The table shows that TgABC transporters digest by different restriction enzymes to give the orientation (right or wrong).

Restriction enzymes	Clones	Right orientation	Wrong orientation
<i>NcoI</i> & <i>Bgl</i> II	TgABC-A(pESC-Ura)	7.2Kb & 2.1Kb	8.7Kb & 0.6Kb
<i>Bgl</i> II	TgABC-B(pESC-Ura)	0.6Kb & 8.1Kb	1.6Kb & 7.1Kb
<i>Bgl</i> II	TgABC-D (pESC-Ura)	1.6Kb & 8.3Kb	0.6Kb & 9.3Kb
<i>SacI</i>	TgABC-E (pESC-Ura)	8.15Kb & 0.85Kb	7.3Kb & 1.8Kb
<i>EcoR1</i>	TgABC-F (pESC-Ura)	8.4Kb & 0.6Kb	7.2Kb & 1.8Kb

TgABC-B, TgABC-D and TgABC-F were successfully cloned in pESC-Ura Yeast expression vector as shown in Fig. 4 and further confirmed by commercial sequencing. There was no mutation observed when sequenced DNA (TgABC-B) and amino acid were

aligned (Sequences Lalign, 2008). The alignment of nucleotide and protein sequence of TgABC-B was given in Appendix IV. TgABC-D did not yield any sequencing and were compared due to some unknown error.

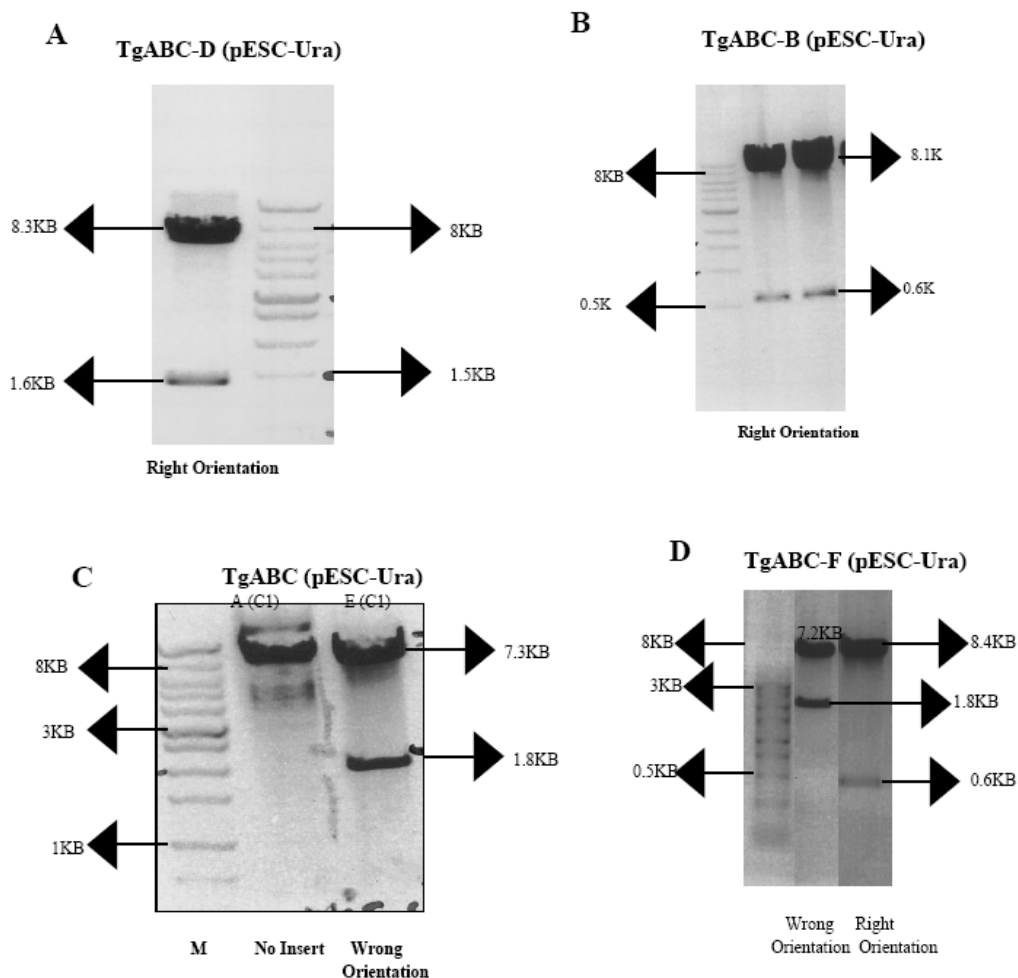


Figure 4: Fragments of different products were shown the orientation of insert in vector (pESC-URA). (A) Clone 1 of TgABC-D digest with *Bgl* II enzyme. The size of digested product was 1.6Kb & 8.3Kb that mean gene is in right orientation. (B) Clone 1 & 2 of TgABC-B digest with *Bgl* II enzyme. The size of digested product was 0.6Kb & 8.1Kb shows that gene is in right orientation. (C) Clone 1 of TgABC-A digest with *Nco* I & *Bgl* II enzyme. The product shows one band due to unknown error. The right orientation of the product was 7.2Kb & 2.1Kb as shown in Table 5. Clone 1 of TgABC-E digest with *Sac* I enzyme. The size of digested product 7.3Kb & 1.8Kb mean gene is in wrong orientation. The right orientation of the product was 8.15Kb & 0.85Kb as shown in Table 5. (D) Clone 17 & 15 of TgABC-F digest with *Eco*R1 enzyme. The size of clone 17 product was 7.2Kb & 1.8Kb means gene is in wrong orientation as well as the size of clone 15 product was 8.4Kb & 0.6Kb shows that gene is in right orientation.

S. cerevisiae AD-18 lacking endogenous ABC transporters (Yeast genetic resource center, 2008) cells was used for transformation of pESC-Ura with inserts as performed by Ito *et al.*, 1983. *S. cerevisiae* strain was designed to test the function of ABC proteins. TgABC-B was cloned in to pESC-Ura and the construct was successfully expressed in *S. cerevisiae* AD 18 strain for drug sensitivity. Due the unavailability of time, the work

could not be continued further. Future research will be needed to clarify the function of TgABC transporters for drug and lipid transport processes.

5 Conclusion

In summary, we identified six genes related to the ABCG and ABCH family in *T. gondii* genome as well as several proteins potentially interacting with them. This number falls in the range reported for other Apicomplexas; 15–20 members in *P. Falciparum* and *P. yoelii*, and 33 in *C. parvum* (Zapata *et al.*, 2002).

The presence of ABC transcripts in tachyzoite and bradyzoite infectious forms suggests an important role of these proteins in the basic biology of this parasite. Further research on membrane transport mechanisms and intracellular biochemical processes mediated by these ABC proteins could result in the identification of novel therapeutic targets. On the other hand, the implication of some of these proteins in the resistance phenomenon in different protozan parasites justifies further investigations on ABC's potential contribution to antiparasitic resistance in *T. gondii* (Klokouzas *et al.*, 2003).

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6 Appendixes

Appendix I

Sequence of TgABC-A (2649 nucleotides)

```
ATGGACGTCGGGCCTCGAGTCGGCGAAGGCGGTTTCGGCGACGGCGACGATCCGAACGAGGACGGCGCGTGGGCGTCT
CTCTACCGTCGGCCCTTTGGGGCGTGACGGTTGTGGTGAGAGACATTGCTTCACTCTGCCTCGGAAACGGGAGTCGA
CGAGATGCAGCTTTTGTAGACGGTGTGCCTCGGCACTTCTGCTCGCTCATCTGCTTCTCCAAGGACCTCCTAACAGTCCCC
CTGCAGCAGCTGGTGGTTGGCGAAAGCCAGCAGGAGAGACGCGAGCTCACAGTACGCGTGGGGTCGACCGGCCAGTGC
CTGTAGACCTGCGAGCGAGATGGATGCGAGCAGCAGCGAGGCGACGCGCATGCGCAGCAGTAGGCTGGCGGCGGAAG
AGAGTGAAAAAGAAAGTGAAGAAGTGGACGGGAAGCTGCGGAAGGCGACGACGAGCCGCGGGCATCCCGCAGAGGA
ACCGGAAGCCGACACACGCATGCAGACCTCGGAAAGCGGGCGCGACGAACGAGGGAGTTCCAAGTCTGGGGGCGGCA
GAGGCGGCCATGGACCAGCTGCGCCGGAACAGTGGCGGGAGGATGAAGCAGCGAAGGACAAACAAGAGGCAGACGA
ACACAGACCGGAAGAACGACAGCTGGTGCTTCATCCCTTCTCCGCGGTGTTGAAGCAGGCACCATGACGGCGGTGAT
GGGGCCGAGCGGGTGGCGCAAGACGACGCTGCTCAACGTGGTGGCGCACCGGACGCAAGCGCGGCAGACTGGCGGCC
AGGTGTTTCGTCACCGGAAGCCCCGAGGTCGCTCGTTCAAGCGCCTGGTGGCTTTTGTCCAGCAAGACGACATCTTTGA
CGGAAAAGAGACAGTGCAGAGAATGCCTCGAGTTTTCACGAGACTTGCGCATGAAGTCTCGTCGATTCCAGACTCCACA
GAGAGACAGCGAGCCGCGGACGCTACACCGACCGAGCCCTGAGGGTTCTAGGCCTGCAGGCTGTGCGCGACAGCCCC
ATAGGAAATGAAGCAGTCAGAGGCGTCTTGGCGGCCAAAAACGAAGGGTCACTCTCGGCCTTGGTCTGATGAGCGAC
GCGCAGATTTTCTTTCGATGAACCGACGACAGGCTCTCGGCTGCGGACGCGTGGCGGTGGTGAGGACGTTGCGG
CGCCTCTGCGACGTGTACACCGTACAGTGGTTGCCGTCAATACACAGCCCTCCATGCAGATTCTGTGCTCTTCTACAA
CTTGATTTTGTCTCTCTGCGAAGGCCGCTGCGCGTACAACGGGAAGGTCGCTGACTGCCAAGCATGGTTCGAGAAGTGC
GGCTTCCCCTTCCCCCTCCACCAAAACCCCGCGGACTACCTCAGCGACCTTGTCTCCCCACACAAGGGACATCTGCGC
AACTCGCCGCTTCTACGAGGCACACCAATTCCCTGCAGTCCAAGCGCGCTGCGCGAGGCGCTGAGGGGTGCCAGA
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CACCCAGCGGAGGTCGCCGGGACAGCGCGGGGCCACGCAGACGAAAACATGCCTCCAGATGGTGCACAGGCTTCC
 AGTTTTCGCGGCTCTCGATTGTCATTGCGAAGCGACGGGAGGTTTCGCTTCTTCTCAGAGGCCGGGGGCGAAGACGCTC
 CACATCTCGCGAGTTTCTGGAAGCAGCTTTCGATGATAGGACGACGAAGCGCGCTCTGTGGCTGCGCGACAGAGGGA
 CGTTGGCGGCGATCTACTGCGACGCCGCGTCGAGGGCGTATTCTCGGCTTGGTACATGGCTCGCGTGGCTCAAACGCA
 GCCGCCGTACTACCAGCTGTCCGCGTTGTTTCTTCTGGTCTACTGTGTTTGGCGTCTGCGCTCTGGACGATCCCGCTCTT
 CGTTCAACAGAAGGCGCAGCTGATTATGGAAGTCACGGGGGGTACTACTCGGCGTTGCCGCACTACTTGGCGACCCAC
 CAGCGTGTCTGCATGCGTCTGGGGGGTCCGACGTCGTTCTTCTTCCATTCTCTGGTCTCGCCGATTTCGAGTGGA
 CGGCGCTGCCCTTCTCGCTCTTCGTCTCACTTCTCGCCTTCTCTGTCGTCGACGGCGCTTCTACCTCGCGTCCATCGCCA
 GCAGCTCGTTCGCTACGCCAACAGCGTCACCGCGGTGGCTTCATGCTCTTACCTTCGTCACCGGCTTCACCACGAA
 CCCTCAGAGCATGCCGCTCTACGTCGGCTGGGTGAGTACCTGTGCCGTTCTTCTCTCGCTTCGAAGCCACAGCCGTA
 CACGTCATGAAGGCGTACCCTTTCGCGGACCAACAGGCTCAGGCCGCGTTCGCACTTTGCCCCGCGGAGAGCCGACC
 CTGGCGTCGGCGGAAGAGCTGTTCAAGCAGTACGCACTAGCTGGAAGGGTGTATGGCGTAACGATGGATCCGGGCAAC
 TACGTGTGGGTTGTGACGCTCCTATTCTCGTCTTCTCGCGTTGCTGTGAAAGGTTCCGCGGCGGTTTTCCAGAGCGT
 CTGGGTCTCGCGCAAACACCGAGAGCACCTGGAGCGGAGTCTGACTCCGAGGTGTGAACAGCAGGCGAAAACGAACG
 AGGAGGAAGCCGGGAGATCGAGCCGCGAAAGTCAAGCTGAGAGCTCGCGGGCGTGGGTAG

Amino acid sequence of TgABC-A (882 amino acids)

MDVGPVREGGFGDGDNDPNEGAWASLYRRPFGACTVVVRDICTFLPRKRESTRCSFLRRCASALCSLICFSKDLLTVPLQQ
 LVVGESQQERRSSQYAWGRPASACRPASEMDASSSEATRMRSRLAAEESKESGRSGREAEGDAAGRHAPAEPEADTRM
 QTSSEGRDERGSSNSGGGRGGHGPAAPEQWREDEAAKDKQEADEHRPEERQLVLHPFSAVFEAGTMTAVMGPSCGCKTTL
 LNVVAHRTQARQTGGQVFVNGKPRGRSFKRLVAFVQQDDIFDGKETVRECLFESRDLRMNFSSIPDSTERQRAADAYTDQA
 LRLVLQAVADSPIGNEAVRGVSGGQKRRVTLGLGLMSDAQIFLCDEPTTGLSAADALAVVRLRLCDVYTVTVVAVIHQ
 PSMQLSLFYNLILLSCCEGRYNGKVADCQAFWENCGFPFLHQNPAADYLSDLVSPHKGHQAQLAAFYEAHQFPAVQARV
 AEALRGAQTPSGRRDSARADADENMPDGAQASSFAALDLHSEATGGSPSSSEAGGEDAPHIASLWKQLSMIGRRSARLW
 LRDRGLTAAIYCDAAVEGVLGLVMARVRQTQPPYYQLSALFLLVYCVCASALWTIPLFVQQAQLIMEVTGGYYALSALPHY
 LATTSVSACVVGSDVVLFSILWFLAGFEWTALPFSLFVSLAFLVVDGAFYLAASSSSFAHANSVTAVAFMLFTFVNGFTY
 NPQSMPLYVGVWSYLCPFLLAFEATAVHVMKAYPFADQQASGRGRTLPAQEPTLASAEELFKQYGLAGRVYGVVTMDPGTY
 VVVVDVLILVLLAVAVKGSAAVFQSVVWVAPNTESTWRRSRLRGVKNQAKTNEEEAGEIEPRKSKLRARGR

Sequence of TgTABC-B (2106 nucleotides)

ATGCCCTCGCATCTCTAATTTGGAGAGTACTACACCGCTGTCCACGACCACAGTGGTGGGCAGCCAACGGAGTCCTTAA
 CTTCATTTGTGAGAAGAAGATTGCAGACTATGCGGTACCGGTGGAGAATTACTGGTCAACTGACCGTCTTTCGCCAGT
 GGCTCTTGACAGAGAGACACGAGCTGTTTCAGAGCCACGCCCATCCCTCTTGCTATGCACTGTCTACCATAGGTAGC
 CGGATCCCGCGGTGAGTGCAGAACTCCTCAGCTTCCAGTGTGGAGGTGCAAGTCCACCTTCGCCTGAAACCGGT
 GCTGCACGAAGTGGGGTTGTTTCAGGTGCTCACACCGCTTCGCGTTGTCACTCTTCATAAGGAAGCCGATGATTCTGTAC
 AGATGAAAGAGACCGAACAAGCGAAGCGTGTGCAAGGCTACAAGTGTTCATACGCTTCACAAACCTCACATTCACTC
 CTCCGCGAGGCGCTTGACAGCCAATCCGTGAAAGAATGGGCTAAAACGGCTTCTGTACACAGTCGTCACGACAGGCAA
 TTGCTGGTTCGATTCTGTTTCGGGACCGTGTCAAAGGCGCTCCTCCGTCCGCATATGCTCGCCCCGACAAATTTCTC
 TATGGCTGAGCGGCTAGCTTTGCCCTGGCGAAATTTGTGGGAATCCTCGGTCTTCTGGAGCAGGCAAAAGCACTTTCC
 TGTCTGTACTGTGTGGAAGGCTCAAAAAAGGAGTCGGCGGACTCATTGATATCAACGGGGAACCTGCCCCAGCGCGAA
 TGAAGAAGATTGTTGGTTACGTGATGCAGCAGGAGTATTCTTCGGAAATTTGACAGTCGAAGAGACACTGATGTACAC
 CGCCAGACTGCGTTTTGGGGAAGAAACCAGTTTCGCAGAGAAAAAGCACGGGTGCAAGAAGTTATAAACTCAATGA
 AGTAGACAAGTGCAGAGGAGTCCGCTGGGGAGCGCTTTTGGCGTGGCCTTCCCGGAGGAGAGCTGAAGCGTCTAA
 ACATCGCGAACGAGCTGTACTTTCATCCGTCACCTTCTCTTGGATGAGCCCAAGCGGACTGGACGCTCGATCTC
 GACCGCCTTCTCGATATGTTGAAACAGCTAACACGAGCCAACAGGTGTACTACCGTCTGCACCATACACAGCCTAAC
 AGCAATGTGTTTCATGAGGTTTGTATCGGGTGATCTTTCTCAAAGACGGCCACATCGTGTACCAGGGGAAGCCCTCCGACG
 TATGGCTGATCTTTCTTCTCTCAATTTCCACTGTCCAGAAAGGTTGGAACATTGCAGATTACGCACTGCATGTCATTAGT
 GCATCCGATGATGAGAACTTCAGGCGGCGTCACTCACCAGCACGACTGCAGCCGGGGGAGATCCGATCACTGGCAGT
 ACACATGGTCTCCTTACTTCACTACTTCTTACTACAGCTTCGGACCTGCCTACATGGCATTACATCTTTCCAGCTGA
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 GCAACAGAGATTAGGCCATGGATATCTTGGTTTCGATGGTTGTCTTCCAGAACTACGCGGTGACAACTTCGTAATA
 GTTACAGTGGGATCGTCTTCGACACTCTCGTCTCGGAGTCTCTTCTGTTCCCACTTGGCCAGGGCAGCCGATCACAG
 CAGAGATGATTACCCACAGGTTCAACACGACACTTCGCTTTATCCCAATATCACCTTAATGATTGTACATGGCTAGCC
 ATCAAATAATGTGCTATGGAGTCTCAAGAAATCTCTAAACTGAAGTCTGA

Amino acid sequence of TgABC-B (701 amino acids)

MPSHPNLESTTPPVHDHSGGQPTESLTSICQKKIADYAVPVENYWSTDRLSPVALDRETTTVSEPRPIPCYALSTIGSRSPAEE
 VRNSSASRCGGASPPSPENGAARSGVVQVLTTPRVVTLHKEADDSVQMKETEQAQKRAARLQVFIRFTNLFTTPRGLARQSV
 KEWAKTASCTQSSRQAIAGSDSVSGPCSKAPPSAYARPRQILFGLSGYFAPGEIVGILGPSGAGKSTFLSVLCGRLLKKGVGGL
 IDINGEPAPARMKKIVGYVMQQEYFFGNLTVEETLMYARLGLKCTSFAEKKARVEEVINSMKLDRGRTVRGSAFCRGL
 SGGELKRLNLANELLHPSLFLLEPTSGLDASISTALLDMLKQLTRANRCTTVCTIHPNSNVFMRFRVIFLKDGHIVYQKG
 PSDVCAFYSSLNFHCPGWNIADYALHVISASDDENFRRLSTSTAAGGDPITGSTHGLLYFTTSYYSFGPAYMAFTSFAER
 AVISRERSKKAYKVSCYLLAKTMDLVVQIFTPSLWLAIVYPLVGLPSDLGVFMFAFWAQLVLLVCIAQAIGQLIAAIVDDAR
 LGLLLSVLISISSSGFYVQQRLGPWISWFRWLSFQNYAVTNFVIVTVGSSSTLSCSEFSFPTCPGPITAEMITHRFTTAL
 SPLSNITLMICTWLAIKLMCYGLKKSLKLS

Sequence of TgABC-C (2946 nucleotides)

ATGGACTTTCTGCGCAGACGCGATGTCTTTCGACGACGAAAGACGCTCGAGAGAAGCGAACTGGTTCTTCTCGCCGA
 GTCGCGAGGGCGGCTCTTCTCGCTGTTCTCTCGCGGGGGGTTTGCATGTGACGCTGCTCGTCCCATCACGCTTGT

GCGCGCGGCTTTTCTACGCCGTTTCGGATGAAGCCGACAGATCCCCTGCACCCAGTGTGCCTCCCCGTGTCTCCCTCGAA
AGAAGCAGAGTAGCAAAGCAGAGGATGCGAACAAGCCGAGCGACGAAAAACGAGCCGTTTCGCGGCGCCGAGCGCTCTC
AGAAGACCCGAGAGCGCAAATTCGTGGCGAAGCAAAGGAGGCCGCGCAGACTCCCCGGCACACGGTGCCTTGGATAT
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CGTCCAAGACGGATCGTCTCTACCTCGGTCTCCAGCCTGGAGAGCCTCTGTCTCAATTGCAACTTACGTCATGCAG
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AAGAAGAGAGAGCAGAGAGGGTGGAGGTCTCTGCGCGAGCTCGGCTTGTGGGGTAGCCGTTTACCCGAGTCTGGCG
GAAGCGCCAAGAAAGGTCTCAGTGGCGGAGAGATCAAACGCTCGCTCTTGCCGTCGAGCTACTTACAATCCGTCTCT
CATTTTCTTGATGAACCAACGAGCGGCCTTGATGCAGCGCTCGCGTTCGAGACCATGAAGCTGCTTCTTCGCTCGCG
CGCCATGGAGGCCGAACCTATTCTTCGACGATTACACAGCCCCGTAGTCAGCTCTTCGCGATGTTTGATCGCTTGGTCTC
GATGTTTCGAGGGGCGCATAGTGTTCAGGGCCCCGCCAGGCACTGCGTATCTTACTTTGCCAAGCGCGGGTTCCACTGC
CCGCTTCAGTTCAATCTCTGCTGACTTCAATCTGAGATCTCCTGAATGTGACAACTTTACTGCGAACACCCCTCTCGCCT
TCCCAGTCGAGGGGTCTCTGAGCGCTCAATCCCTCTCGAGGCGGAGTTGAAGAAGGTCCAGACGCAAGAGCTGCA
GCGGCTCGCACTCGCGACGCGGGAGGGCTCGCGACACGAGGCCTGGGGAGCCCCAGGAAGTCCGAGGCAGGTCTG
GCGAGCTTTTGGAGCCAGTGCCTCGTCGGACGGCTTACTCCGGGCGCGAAAGGCCCCAGGCGACCGCAGCCGAG
GGACAGATGCCGAAGAGACCCGCGAACGACCCGAGAGAGACGCGGGGGACCCATGGGGGCGGCAGCGGGCAGGGC
GGGAGATCAGGAGGAAGAGACAGGTCCGCGTCACGAGGCTAGTCCGGCGCCCTGGCGAGACATCGTCCACGGCC
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ACGATGTGAAGCGGCTAGCAGACTCGTATGCGGCGAGGCCAGAGAGAGCGCATGTCGAGGAACTGATCGTTCAGTGT
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GGTCCGACTGGGGCCGGGAAATTTGTGTCTCATTCAAATGGGTTTCTAAATCATGTCAGGAATCCAATGAGTTCTGT
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TCTGCCGATCGCCGCGGTTGAGTTGCCAGGCCGTCGCTCCCATCATTCTTCTATTCTTCTCTGTTTACGGGCTA
CTACATTGCGCTGATGACATTCCAGCGGTATCCGGTGGCTGAAATACCTGTCGCCTATTCACTATTCTGATGTGGCTC
TAGCTCTCAATCAATTTCCGCTGACGAGAAATGGGGTATTCTGTCACCAAGGACTTTCTCGAGTCTACGGAGGCAT
CACTAAGACAGATCTTGGGTTCTACATTGGCATGCTTGTCTGCTCGGCTGCGTTGCCGCGTCTGTCTTCTTCTGTCT
GAAGTACATGCACAGGCGCATCGGCCTTGAAGCGTGA

Amino acid sequence of TgABC-C (981 amino acids)

MDFLRRRDVFRSSKDAREKRTGSSSPSREGSSSLFSPAGGLHVTPARPITLVARGFSYAVRMKPQIPCTQCASPLPRKKQSS
KAEDANKPSDENEPFAAPSLRRPESANSWSKGGRADSPAHGALDIEKGSEDOHIGDCA TENRTPEERSADGETETEGPLKI
ILKDINLCARPGEMLVIMGPSGSGKTTLLNAFAGRSPSSRVVDQGSLLYLGLQPGEPLSSIATYVMQKDMVPELLTVQEYVT
FFSRLKMRDATEEERAERVEVVLRELGLWGSRFTRVGGSAKKGLSGGEIKRLALAVELHNP SLIFLDEPTSGLDAAAFET
MKLLRLARHGGRTILCTIHQPSQLFAMFDRLVLMFEGRIVFQGPARGVSVYFAKRGFHCPPQFNPA DFI DLLNVTTLTAN
TPLACPQSEGLSAQSLLEAELKKVQTQELQRLALATREGSRHGGLEGPEAPRQVGELLEPSASSDGFTPGAERPQATAAA
GTDAAETRERPERDGGGPMGAAAGRAGDQEEETGPRHEASAPLARHRSHGQESGASGRRRRRGNDEADTPGGS AQDGSVE
GRKVSVSVPIVSAPSSEVVEANGEDAGGARA AVSQGDARDKPRQSRLQGEARIASGFLDAEETQGELHRVLVDENDVKRLA
DSYAASPERAHVEELIVQCLQAAPPDHVKPSGRAAAVKRLLPQRRWSDWG REICVLIQMGFLNHNPMSSVVQLALNIFL
GLIFGAIFFNIPQGQTIDSARNMLGCLFFLGSQ LIFGLDCLVLFCTNREL FN RD TANGNYTPFSYFVAKCLSNLPFEHLPLTC
VTLVAYGMCGLHRGA AHFFIYFVIGQLSIFASTSLGLISAASPRVAVAVAPIILLIFLLVTGY YIRADDIPAVIRWLKYLSP I
HYSYVALALNQFPPEDEKWDSSNKDFLESYGGITKTDLGFYIGMLALLGCVCRVLSFFCLKYMHR RIGLEA

Sequence of TgABC- D (2301 nucleotides)

ATGGCAGACTCTGCCCTCGCACCTCAAGAGTCTGCCGCACTGTTGCCGCTGACAATGCCGCTTCTTCCAGGGAGGGAG
GTCGAGGTACGTCCGGCGCGCATGGACAGGGCGCGCGCGACTGCAGAGCGGCGACTTTCTCTGGACGGCGCGACCG
CGGAGCCGAGCAGTGGAGGCGCTCTCCGGCCGAGTCGGACGCGGACATGCTCGAAAGAGGCCTTCTCGGAGCTTCCG
CCTCTCCGGAGGCTCGCGCATGCAAGGACGAGGCAGTCGATAACTCGCCAAGGCCGCGCAAGGAAAAGGAAGGTGCC
CCCTGCTCCGAGCCTCTCCGTCTGTCGAGAGATTGGAGTCTCGTGGACGGATATCTCCTTTGAAGCGCGGGTGCGAA
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TCTGAAAAGAGATTTGTGCGGCGTCAGCGGAGAGTCTTCCCGCGATATGGTGGCTCTGATGGGCGCAAGTGGAGC
AGGAAAGTCGACGCTGTTGAACATTTTGAGTCGATATTTCCGAGAGACCTCTGGAACGGTAAAGTACGGAGACGCGCA
GTTGGAGTTGAAGGAGGCGAAGAAGGTTTCTGCTTCATTTCAGCAAGAAGATCTCTTCAACGGATTTCATCTGTTCTGA
GAGCATCTCCAGTGCATTGTTCTGACTCCGAACACGCTGCCGCCGAAAGAGCGCGAGGCTCTCGTCGACCGCCTCTCG
TCGCTTTGAACTTTGAAAGCGGCAGACACATGCATAGGCAATCTTCAGATGGGAGCGCGAAGAGGAATCAGTGGAG

GCGAGAAGAAGCGCCTGAGTGTGCGGACGGAAATCGTGACAAATCCTTCCATCATTTTCGCGGACGAACCGACGACAG
 GTCTGGACTCGTTTCATGGCCGAGGCTGTGATGACTGTTCTCGAGCGGCTCGCCAAAAATGGGCGTTTCGATCATTTGCAC
 AATCCATCAGCCGAGCAGGACGGTGTGAGAAATCAACAAAGTGATTCTGCTCGCAGAAGGCCGATGGTCTTCGCT
 GGAGACCGACTCGCCCTGCGAGTGTATTTGCGCGCGCTCGGAAAGTCGATTCCGCCCTACACCAGTGTGCTGACTTCG
 TCATCGACGTCCTCTCTCCAGCGAAGGCGCAGAAGCGACTCTCCAAATCGCGGAGAAGATGCATGCGGCGTGGATCA
 ACACAGGCGTTCCTTCATGCGTGACTGGCACGAATCCCTGCGAGAGCAATACATCCAGAGGCTGCAGTCTGAGGGAG
 TTGCGGACAACTCAGTCTGTTCAAGGGCGAGACGAAGCGTCTCGCTTTGCGCAAGAAGCGCTGGAGGCTCCGACTG
 CGACTGATGAGGAGCCAGGTCTCAAGAAGAAGGAAGCCGAGTGGAGGGTTCGCGTGAGTTGGTGACGCAATTCCAG
 GTGCTGCTCCACCGCGCTCGCTGGCGAACAACGAAATCCACAGATTCTGCAAGCTCGTGTGGGCCAGACTCTCGTCT
 CTGCTCTTCTCCTCGGCTTATTTTTCTTCGCTTCGTAAGGGGATGCCATTTCGAAGAACGGCGCTGCCAACTTCATC
 AACCTGAATCAGGGCATGACAGGTCTGGTCACCGTTCTGCGACGTTTACGACGGACAAAAATCGTCGCGCTGCGCGAA
 TATCGCTCGGGGACGTACAGCCTCGTTCCGTATTTCTGGCAAAGACGGCTGCCGACGCGGCGTTCCAAATTTTTAACC
 CAGTCGTCTTCTCAGCATCGCATGGTACATGATGAATCTGAATCCGTCGGCGACCCGCTGGCTCTGGGGCCTTGCGTTC
 ATCTCTCTGCAACGCAACGCGTCGATTTCGATGGGCTACTTGATCTCGTGCATGTGCCCCGACCTTGAAATTGCCCTCTC
 GGTCATGCCGCTCTGACGATGCCACTAATCCTTGTGCGGGATTTCATGATCATTCTCGACTCTCTGCCAGATTCTGGA
 TATGGGTGCCGTATCTCTACCCCTTCGGTGGGCTTCTCCGGCATCATGCACGCCGTCTGGGAAGACGTCGAACCTCGA
 TCCTTGGCCTGCGGGCATGAATCCGCCGCTCTGCTACAGCTCAGGCGCTGAGGTTCTCGAGTACTACTGCCTTGATGGC
 GACAGCATGTGGCTGAACGCTCTCTACCTCGTGATCATGGTCGTCGGTACCAGCGTTGTGCGCCTCTTGTTCTCCTCAT
 CTGAATCGCCGAACTAA

Amino acid sequence of TgABC-D (766 amino acids)

MADSLAPQESAGTVAADNAASSREGGRGTSAGHGQGARATAERRLSLDGATAEPSSGGALRPQSDADMLERGLLGASAS
 PEAAACKDEAVDNSRPRKEKEGAPCSEPLRRCREIGVSWTDISFEARVRRPTGILGTPPLCDVLNALDSLQKGPLKKILCG
 VSGEVLPGDMVALMGASGAGKSTLLNLSRYLRETSQVYGDAAQLELKEAKKVSCFIQEDLFNGFITVREHLQCIVRLRT
 TLPPKEREALVDRLLVAFELSKAADTCIGNLQMGARRGISGGEKKRLSVATEIVTNPSIIFADEPTTGLDSFMAEAVMTVLER
 LAQNGRSIICTIHQPSTTVFEKFNKVILLAEGRMVFAAGDRLALRVYFARVGKSIPPYTSVADFFVIDVLSSEGAETLQIAEKM
 HAAWINTGVPMRDWHESLREQYIQLQSEGVADKLSSFGETKRLALRKEALEAPTATDEEPGLKKKEAAVEGRVSWWT
 QFQVLLHRRSLANKRNPQILQARVGQTLVSALLLGFIFLRRLKGDASKNGAANFINLNQGMTGLVTVLQTFITDKIVALREY
 RSGTYSLVPYFLAKTAADAAFIQNPVVFSSIAWYMMNLNPSATRWLWGLGFIFLQTNASISMGYLISCMCPDLEIALSVMP
 LTMLPLILVAGFMIILDSLPRFWIWPVYLSFPRWAFSGIMHAVWEDVELDPCPAGMNPSPCYSSGAEVLEYCYCLDGDSMWLN
 ALYLVIMVVGVRVVGLLVLLILNRRN

Sequence of TgABC-E (2439 nucleotides)

ATGAATTCCTCGCTGGCAGCGTCTGCGGGCAAGGCCAGCCTGTTGAAGAGACAGGAGTGGAGGCCCGGCCTCTGAC
 CCTTTGAGATGGAGAGAGACACCCAGCCGAGAGGAACCTTCTGGTGCAATTTTCTTCTCAAGGCGTCTCACGCG
 CGGTGTGGAGAGTCGACGGAAGAACAAGTTCACCTGCTCAACGACGGAGTGTCTAGACACCCCGGGGACTTCCGG
 ATGCAGAGGGCTGGGATGAGAATCCAGTCCGAGTCGACACAGGCGACGAGTGGCTCATTACGACGGACTCCCCCTCG
 TCACCTCAACTTCGAGGACCTCACATGGACGTCGCTGACTCGGGCGACGGCTGCGTGAAGAGAGCGTTGAAGGGTG
 TCTTCAACCGCCGATCAAGGAGCCGACGCGAAAAGAGAATTCTCTCCCTCGAAGGCCTGAAAAGTTCCTTCCGGCCTG
 GAGACTGTGTCGCGCTGATGGGAAGCAGTGGAGCAGGCAAAACGACTCTTGAATGTTCTGTGCGGGCGCGTGACGA
 AGAATGTCGGAGGCCGCGTTCAGTACAACGGTCTGGAATTGCCTCCTGAGGCACTGAAGGCAATTTCTTGCTTCGTTCA
 ACAAGAAGTGATTTTCTCGGAACCTTGACCGTGCAGGAGCATCTCGAGTACCAGGCTGCTCTACGGCTGCCGCTTCG
 CTCTCTGCTCGTGACCGCGCAGCGACAGTGAACGCGATGATTGAAAAAGTCGGTCTCTCCAAAGTCGCGGATTCCCTTA
 TTGGCAATGTCTCTCAGCAGCAGCTCGTCGGCATTTCCGGAGGCGAACAGCGACGCCTGTCTGTGGCGACGGAGCTTCT
 GACAGAGCCTTGTGCATCTTCGCCGACGAACCGACCGGCCTCGACTCTTACATGGCGATGCAAGTCGTCAAGCTT
 TTCAAAGGGCCTCGCTCTCGACGGTCAACTGTCGTTCTGCACCATTCACAGCCAGCTCGAGTGTCTTTCGCGAGTTCA
 ACAAGGTGCTTCTGATGTCGGAAGGGCACCTTCTGTACTGCGGAGACCGCGAGGCTTGCATCGGTTGGTTTCGCGCATTT
 AGGGCAAGTTTTCGAGGCGGACATGAATCCAGCTGAGTTTTTGATTAAAGTCACTGCAGTGACGGACGACAATCGCGA
 GGCGGCTGTCCAGAGAACGGTGGAGTGGGCTGAACGTTGGCGGCAGGAAGGCGCTATGTTTCTCGAGCAGTGGGAGGC
 TCTTGGCGGACGCGTCCGCTTCTCCGATCAGATGCGCATTCAGCGCTTTTTCATCGATGGAAGAACCGGCTGAG
 GCCTGCGCAAGGAGGCTCCAGGTGAAAACGAGAGGTCGCGGGGGTTACGAGAGCGCTAGTCGAGACCAAGCAGCC
 TGGAGAAGTCGATACATGCGGTCTCTGTCGACAGAGCGCCAGACGTCGAGAGCGGATCGGCCGGAAACGCAGAA
 CGCCGAGCCGACGCTCTTGATCGAAAACCTGAGTTCCGCGCGCCAGTACATGCTGAAGGGCGGTGCCATGTCTGAAGGC
 TTGTTTGGAGGAGATGAAAACAGACAGAACTCGGAGTGTCTCAGAGAAACCTGGCTGCAGATCCAACGCACTACTCTGCT
 CAGAGGACGCGACCCCTTCTCCACGTATGTCCGACTGCTCACAAACCGTGATCAGCGCTCTCATCCCTGCCCTCATGTAC
 TACCGCTTGACGTGGCAGAGCTCAGACGCGTGGAACAAAGTATCTTCTTTTACATCATCCTCAGCGAGTCGATGG
 CTTGTCTCTTCGGCGCTTCCATGGCCTTCAACAAGGAACGCGCTGTATCCAGCGGGAATACGAGTCCGGAGTGACACG
 AATGCCTCTCTACTTCATCGGCCGCATCACCGCAGACTCCCTCCTCTGGATGTTTTTCCGTTTCATCTATCATTTAATCGT
 CTACTGGATCTCCGATCTCGGGGAGACTCTGTGAGCAAGTACTTTGCTTCCCTCGCGATCACGCTGCTCCTGATTCAGG
 TTGTCTCTCTACACCTACGTCGTCGTCGCGCTCATCAACATCCAGTTGCGTCGACAGTTGTTCTTCAGATCATGCAG
 ATGATCCTCACTCTTCTCTGGAATCATGGTCAAGTTGGACGAACTGGGAAAGTTCTGGATCTGGATTGTGTACCTCTC
 GCCATTCAAGTACGCGCTCCCTTGCTTACCGTCACTATTTTCTGGAACACAGAAATCTCGTCGCCCTCAGGATCCACCG

TCTCCGGCGTTGACTTCCTGAACGATACCTTCGGATTTCAACATGACAAGTTCTGGCTGTACGTCGGCCTGCTATTTCGTC
CTCGGAATCAGTGGGAGGCTGTTGGGAATGTTGCGCTGTCGTGGAAGGCGAGTGAACGAAGGAGAACCAATGA

Amino acid sequence of TgABC-E (812 amino acids)

MNSLAGSVVVGQGPVEETGVEAPASDPLADGERHPSREEPSGAIFSSQGVSRVSVESRTEEQVHLLNDGVSRRHPGGLPDAEG
WDENPVRVDTGDAVAHYDGLPLVTLNFEDLTMDVVTGPDGCVKRALKGLFKRPIKEPTRKRLSLEGLKSSFRPGDCV
GSSGAGKTTLLNLVSGRVTKNVGGRRVQYNGLELPPEALKAISCFVQQEVIFGTLTVQEHLEYQAALRLPPSL
SARDRAATV
NAMIEKVGLSKVADSLIGNVSQQQLVGISGGEQRRLSVATELLTEPCVIFADEPTSGLDSYMAMQVVKLFKGLALDGR
TIVVCTI
HQPSSSVFAQFNKVVLMSSEHLLYCGDREACIGWFAHLGQVCEADMNPAEFLIKVTAVTDDNREAAVQRTVEWA
ERWR
QEGAMFLEQWEALGGRAAASSDQMRIQRLFSSMEEPAEACAKEAPGENGAGPGGSGALVETKQPGEVVSHAVLVDQ
SAQT
SEADRPETQNAEPTLLHRKLSSRAQYMLKGGAMSKACLEEMKTDRIQVLRVETWLQIQRSTLLRGRDPFSTYVR
LVTTVISAL
IPALMYRRLTWQSSDAWNKVVSSSYIILSESMACLFASMAFNKERAIVIQREYESGVTRMPLYFIGRITADSL
LWMFFPFIYHL
IVYWISDLGGDSVSKYFASLAITLLLIQVVLSYTYVVVALIKHPVASTVVLQIMQMLTLFSGFMVKLDELGKFW
IWIWVLS
PFKYALPCFTVTFWNTISSPSGSTVSGVDFLNDTFGFQHDKFWLYVGLLFVLGISGRLLGMVALSWKASRTKENQ

Sequence of TgABC-F (2385 nucleotides)

ATGGACGCTCCGGACATGAGGCGGGGAGCTCGTTGGTCTGTCGACTCTGTGCTTCCCCCTCGGGTGGTGGCTCTTCCG
AAGCCGGCAAGAAGGAGGACGGGGAGGCTGGGGATCCTTCTCTGCTTCGCTTGCAGAACGAAGAGACGGCCGCGCC
TTCAGCGGCGCAGAGACTTGGCGGGAGGCGAGGGGACGCGCTTCTCTGCGCTTCCGCGGCTGGCTGTGGAGACAGCCA
GCGAGAGAAGAGATCTCGAAGCGGCCGGGAGGGCAGAGGAAGAGCGCGAACTCGGCCGGCTCTTGCCTGCATGCGA
GACGCCAGGTCGAGTCTGCAAGGCGTGCACTGCCCAAAAAAGGCAACCTGCTCAAGAGAGATCGCCAGACCG
ATGCAGAGCGGTGGGGACGAAGTCGAGGCGTGTCTTTCTCCTTCAGAGACGTCCAATGCAGCGTCCGCTTCGCCGCCCC
CGACGGAAAAAACAGAAGAAAACGATTCTGTGGCCGATCTCGGGCGACTTTCTCTCAGGAAGCGTCGTCGGAATTCT
CGGACCCTCTGGAAGTGGAAGACGACGCTTCTTGACGTCTTGGCGAGTAAAAAAGTCCCCAAGGCCTACACCGGCGA
GATCTTTGTGACGCGCAAGAAACGAGAGGATAAACGCTTCAAAGTCTCTCCATCTACGCTCCTCAGGAAAACATTTTC
AATGGAACGAAGAGGTTCCGGGAAGTGCTGGCTTTCGCTGCGGCTCTGAAGTCGGGTCTGTCGAAGGAGGATCAGAAG
AGGCTGGTGGACGCAACTCTTTCTTTCTCGGTCTTGAGCGCGTTGCTACTCAACGCGTTGGAAACACCGTCGTTCCGG
GCATCTCCGGAGGCCAGAAGCGTCGTCATCGGCCGCGCATTGGTCACCAACGCGAGTCTCGCCTTTTGGCAGCA
ACCGACTTCAGCCTGAGCTCGACGACGACGAGGCGCTGATGCTTGGCATCAGGGAAGTGGCGAAGGCCTGTGCGCT
CACCTTCTCATCGTTATCCACCAAGCCGCGTGTGGAAGTCTTTGAGCTTTTCGACGAGGTTATCTCTCCGCAACAGGCC
GGTGTCTGTACAACGGCCACGGTCCCAGATGGAAGCATACTTCTCTGCCCTAGGCTTTCTCTTCCACCCTACGTGAAT
CCGGCAGAGTTTTACATGGAGCTCACCGCGCACGACGCGACTGTGGAGCAACTCGCGACCGCGTACGCCTCGAGAGCG
GAGACAGGTGTCTCCTCGCGCCCAGAGGCGCGGCTTCCGGCTCCCTGCTGGAGGACGCGCAGAGACAACAGCGAGA
AGAGCTGGCGGAGGAGGTCGACGGTGCCGACGCGGACAGCAAGAGGAAAGAGGCGAACACCTTGTGGAGAAAAAC
GAAGAAGACGAAACGCCGAACAGTCGAAGAGAATCTACAGCAGCGTCTCTCGCAGGCATGCACGCGCTCGTGACGGC
ACTTCACGACGATCTCGATCGATGCATCCACTCCCGGGATTCTTCGGACGGTTCATGATTTTGACGCGTCGCAATGTG
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TCTTCTCAACGTCTACCAAGAAAAAGCCATCATGTATCACCTGTCGGTCTTCTTCTTGGTTTCGCTTCTCTGGCAAGC
ATCCCGGCGGTGAACATGGCGATGTATCTCGAAAAAGAAGGTGTCTTATCTCTACGAGGCCTCCGACGGCTTTTACACCG
CGGGGCCGTACCTTCTGTCTGAAATGACCACCGGCTTCTGATGCTGCTGGGCGCGACGCTTCTGGTCTTCGTCGTGGT
TTCCCTGCTGCGCCTTTCCGTTTTCCAAATTCGGGATGGTCTTTCTGCTCTTATTCTTTTCTCTCGTGGTGGACGCC
GTCATGCAGCTCGCTGCTGCCATGTGCGCCACTTTCATTATGGCGAGCACCTTCGCGGGCGGCTGGCTGGCGTTGACTT
CCGTGTGTAATGGGTACAACGCAAATCCCAAGTCGATGCCTGCATGGCTCACGTGGCTGGTGTACCTCTCGCCCTTCTA
CTACCTCATGGATGGTGTGGCGATTGTCTCTGCTGGGAGAACCAAGATCTTTTCGGAACCTCCAGAAAAAGCAGCGAA
ATATGGATACACCAAGTTGTGAAGAACTTCTGAAGGCTTACGGGTTTGTGGGACCCTCGGGGAAGCTCCCTGACTCCG
CCACAGTGGCTCTGGTCTGTGGACGTCCCAAGTTTGTGCTTAATGACTGTCGGCATGAAGACGTTCCGCTGTTTTTACCA
GGCGTACTGTGTCCGGCTTCGTCGCTGA

Amino acid sequence of TgABC-F (794 amino acids)

MDAPDMRRGARWSSDSVASPSGGGSSEAGKKEDGEAGDPSSASLAERRDGRAFSGAETCGRRGDALLSRFRGWLWROPAR
EEISKRPGGQREERELGRLLPACETPRSESSEKACSPHKKGNLLKRDQTDARWGRSRGVVSFRDQCSVRFAGPDGKKQK
KTILWPISGDFPPGVSUVGILGPSGSKTTLLDVLASKKVPKAYTGEIFVDGKKREDKRFKALSIYAPQENIFNGNEEVREV
LAF
AAALKSGRSKEDQKRLVDATLSFLGLERVATQRVGNTVVRGISGGQKRRLVIGRALVTNASLAFCDPTSGLSSTDAEALML
GIREVAKACRVTLFVIVHQPRVEVFELFDEVILLAQGRCLYNGPRSQMEAYFSALGFPLPPYVNPAYFYMEHTAHDA
TVEQLA
TAYASRAETGVSSRPEAAASGSLLEDAQRQOREELAEVVDGADADEEERGEHLVEENEDETPNSRRESTAASLAGMH
AL
VTALHDDPRSMHPLPGFFGRFMRTRNVTLAWRDRNVFMVACNALLVAMLASVLFNVYQEKAIMYHLSVFFLVLSLA
SIPAVNMAMYLEKKVSYLYEASDFYTAGPYLLSEMTTGFLMLLGATLLVFVVVFPCAFPFKFGMVFLFILFLLVVD
AV
MQLAAAMCATFIMASTFAGGWLALTSVNGYNANPKSMPAWLTLVYLSPFYVLMGVAILVCWENQDLFGTPEKAAK
YGYTSCEELLKAYGFAAGTLGGSSLTTPQWLWSVDVPVLCMLTVGMKTFACFYQAYCVRLRR

Appendix II Restriction summary results

TgABC-A

#	Enzyme	Specificity	Sites & flanks	Cut positions (blunt - 5' ext. - 3' ext.)
1	AclI	CTGAAG(N) ₁₄ NN	list	1598/1596
2	BamHI	G GATC	list	2418/2422
3	BbsI	GAAGACNN NNNN	list	1637/1641
4	BfuAI	ACCTGCNNNN NNNN	list	*330/334
5	BsaAI	YACGTR	list	*2433
6	BseRI	GAGGAG(N) ₃ NN	list	2602/2600
7	BspHI	T CATGA	list	2279/2283
8	BspMI	ACCTGCNNNN NNNN	list	330/334
9	BstFI	R CCGG	list	*303/307
10	BstGI	T GTAC	list	1181/1185
11	BssSI	C ACGA	list	890/894
12	BstBI	TT CGAA	list	*2258/2260
13	BstYI	R GATC	list	2418/2422
14	EagI	C GGCC	list	*2492/2496
15	MluI	A CCGC	list	*290/294
16	NcoI	C CATG	list	552/556
17	NruI	TCGCGA	list	*1648
18	PfiFI	GACN N NGTC	list	*1375/1376
19	PfIMI	CCANNNN NTGG	list	2009/2006
20	PpuMI	RG GWC	list	219/222
21	SacI	G AGCT	list	2634/2630
22	SbfI	CC TCCA GG	list	992/988
23	Tsp509I	AATT	list	1432/1436
24	Thl111I	GACN N NGTC	list	1375/1376

TgABC-B

#	Enzyme	Specificity	Sites & flanks	Cut positions (blunt - 5' ext. - 3' ext.)
1	Acc65I	G GTAC	list	*114/118
2	AclI	GT MKAC	list	223/225
3	AfeI	AGC GCT	list	*978
4	AgeI	A CCGG	list	*117/121
5	AvrII	C CTAG	list	1749/1753
6	BbsI	GAAGACNN NNNN	list	1901/1905
7	BdaI	NN (N) ₁₀ TGA(N) ₂ TCA(N) ₁₀ NN	list	#1197/1195+1231/1229
8	BglI	GCCN NNN NGGC	list	1471/1468
9	BglII	A GATC	list	1586/1590
10	BmtI	GCTAG	list	2050/2046
11	BsaAI	YACGTR	list	*807
12	BseYI	C CCGG	list	*777/781
13	BsmI	GAATGACN	list	1474/1472
14	BspEI	T CCGGA	list	*997/1001
15	BspHI	T CATGA	list	1190/1194
16	BstBI	CCGCTC	list	*641
17	BstFI	R CCGG	list	*117/121
18	BstAPI	CCANNN NTGC	list	109/106
19	BtgZI	GCGATG(N) ₁₀ NNNN	list	*1008/1012
20	BtsI	GCACTGNN	list	1321/1319
21	DrdI	GACNNNN NTGC	list	1579/1577
22	EaeI	Y GGCC	list	*1224/1228
23	EclI	GCGCGA(N) ₁₀ NN	list	*761/759
24	EcoNI	CCTNN NNNAGG	list	476/477
25	FalI	AA(N) ₅ AAAG(N) ₅ CTT(N) ₁₀ AA(N) ₅	list	282/277+314/309
26	FauI	CCCGGNNNN NN	list	*252/254
27	FspAI	RTGCGGAY	list	*1263
28	FspI	TGCAGCA	list	*1263
29	KasI	G GGCC	list	*588/592
30	KpnI	GATAC	list	118/114
31	MfeI	C AATT	list	548/552
32	NarI	GG CGGC	list	*589/591
33	NciI	CC GGG	list	*1392/1393
34	NcoI	C CATG	list	1831/1835
35	NdeI	CA TACTG	list	606/608
36	NheI	G CTAG	list	2046/2050

TgABC-C

#	Enzyme	Specificity	Sites & flanks	Cut positions (blunt - 5' ext. - 3' ext.)
1	AccI	GT MKAC	list	*1946/1948
2	AclI	CTGAAG(N) ₁₄ NN	list	2929/2927
3	AflIII	A CRYG	list	2285/2289
4	AhdI	GACNNAN NGTC	list	22/21
5	AlwNI	CAGANN CTG	list	#1142/1139
6	Apal	G GGCC	list	#1132/1128
7	BaeGI	G KGC	list	#1132/1128
8	BciVI	GTATCC(N) ₁₀ NN	list	1158/1157
9	BglI	GCCN NNN NGGC	list	*1028/1025
10	BglII	A GATC	list	2838/2842
11	BmtI	G CTAG	list	1972/1968
12	BpuEI	CTTGAG(N) ₁₄ NN	list	748/746
13	BsaWI	W CCGG	list	2702/2706
14	BseYI	C CCGG	list	*559/563
15	BsmI	GAATGACN	list	592/590
16	BstFI	R CCGG	list	*2105/2109
17	BssSI	C ACGA	list	1600/1604
18	EaeI	Y GGCC	list	*1641/1645
19	EcoNI	CCTNN NNNAGG	list	1297/1298
20	EcoRV	GATATC	list	*390
21	FspI	TGCAGCA	list	*13
22	HincII	GTY RAC	list	*1947
23	MfeI	C AATT	list	683/687
24	NheI	G CTAG	list	1968/1972
25	PciI	A CATG	list	2285/2289
26	PfiFI	GACN N NGTC	list	712/713
27	PspOMI	G GGCC	list	#1128/1132
28	PspXI	VC TCGA	list	*1297/1301
29	PvuI	CGAT CG	list	*1760/1758
30	SacI	G AGCT	list	827/823
31	SacII	CCGC GG	list	*2605/2603
32	SalI	G TCGA	list	*1945/1949
33	SgrAI	CR CCGG	list	*2105/2109
34	SphI	G CATG	list	2862/2858
35	StuI	AGG CCT	list	*1379
36	Thl111I	GACN N NGTC	list	712/713

TgABC-D

#	Enzyme	Specificity	Sites & flanks	Cut positions (blunt - 5' ext. - 3' ext.)
1	AfeI	AGC GCT	list	*1394
2	AflIII	A CRYG	list	1901/1905
3	AleI	CACNN NNCTG	list	#1039
4	AflI	NN (N) ₁₀ GCA(N) ₂ TGC(N) ₁₀ NN	list	797/795+831/829
5	BbvCI	CC TCA	list	*2171/2174
6	BglII	A GATC	list	675/679
7	BmtI	ACTGGNNNNNN	list	1798/1797
8	BsaXI	NNN (N) ₁₀ AC(N) ₅ CTCC(N) ₁₀ NNN	list	1174/1171+1204/1201
9	BstHKAI	GLWGC	list	#1036/1032
10	BsmFI	GCGAC(N) ₁₀ NNNN	list	*1749/1753
11	BsmI	GAATGACN	list	404/402
12	BspEI	T CCGGA	list	*240/244
13	BspHI	T CATGA	list	#2009/2013
14	BspQI	GCTCTTCN NNN	list	1577/1580
15	BstFI	R CCGG	list	*34/38
16	BstAPI	CCANNN NTGC	list	10/7
17	BstBI	TT CGAA	list	*799/801
18	BstEII	G GTNAC	list	1674/1679
19	BstYI	R GATC	list	675/679
20	BtgI	C CRYG	list	155/159
21	BtgZI	GCGATG(N) ₁₀ NNNN	list	*1806/1810
22	EagI	C GGCC	list	*184/188
23	EcoNI	CCTNN NNNAGG	list	1654/1655
24	EcoRV	GATATC	list	366
25	FspI	TGCAGCA	list	*1384
26	MluI	A CCGC	list	*1901/1905
27	MseI	T TAA	list	1799/1801
28	MslI	CAYNN NNCTG	list	#1039
29	NaeI	GCC GGC	list	*36
30	NciI	CC GGG	list	*512/513
31	NgoMIV	G CCGG	list	*34/38
32	NruI	TCGCGA	list	*886
33	PfiFI	GACN N NGTC	list	*1174/1175
34	PfIMI	CCANNN NTGG	list	2039/2036
35	PshAI	GACNN NNCTG	list	1561
36	PstI	AA(N) ₅ (N) ₁₀ GAAC(N) ₂ TAC(N) ₁₀ AA(N) ₅	list	*2209/2204+2241/2236

TgABC-E

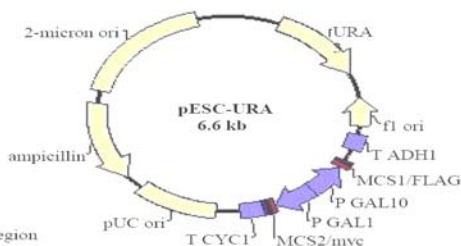
#	Enzyme	Specificity	Sites & flanks	Cut positions (blunt + 5' ext. + 3' ext.)
1	AarI	CACCTGCGNNNNNN	list	200/204
2	AclI	AA ⁺ CGATT	list	*1212/1214
3	AdIII	A ⁺ CRYGAT	list	1751/1755
4	AluI	GACNNNNNNNN	list	207/206
5	ApnI	A ⁺ (N) ₅ (N) ₅ GAAC(N) ₅ TTGG(N) ₅ (N) ₅	list	1555/1550+1587/1582
6	AleI	A ⁺ (N) ₅ (N) ₅ GAAC(N) ₅ TCC(N) ₅ (N) ₅	list	*548/543+580/575
7	BaeGI	G ⁺ GGCN ⁺ C	list	1049/1045
8	BamHI	G ⁺ GATC ⁺ C	list	2275/2279
9	BanII	G ⁺ RGCT ⁺ C	list	1747/1743
10	BclI	T ⁺ GATCA	list	*1694/1698
11	BclI	A ⁺ NN ⁺ (N) ₅ TGA(N) ₅ TGA(N) ₅ A ⁺ NN ⁺	list	1131/1129+1165/1163
12	BglII	CACGTC	list	*1736
13	BpuEI	CTTGAG(N) ₅ NN ⁺	list	125/123
14	BsaAI	YACGTR	list	*1670
15	BsaBI	GATNNNNNNNN	list	254
16	BsuFI	GGGAC(N) ₅ NN ⁺	list	239/243
17	BspDI	AT ⁺ CGAT	list	*1315/1317
18	BstDI	GCAATGANN	list	795/793
19	BstFI	R ⁺ CGGGY	list	*1326/1330
20	BssHII	G ⁺ CGGC ⁺ C	list	*1527/1531
21	BssSI	C ⁺ ACGAG	list	713/717
22	BtgI	C ⁺ CRYGAG	list	1823/1827
23	Clal	AT ⁺ CGAT	list	*1315/1317
24	EcoNI	CCTNN ⁺ NNNAGG	list	2038/2039
25	FauI	CCGCGNNNNNN	list	*1850/1852
26	FspAI	RTCCGCAAT	list	*1294
27	HindIII	A ⁺ AGCT ⁺ A	list	937/941
28	MluI	A ⁺ CGGCT	list	*1751/1755
29	NcoI	C ⁺ CATGG	list	1823/1827
30	PfFI	GACNNNNNNNN	list	*475/476
31	PpuI	A ⁺ (N) ₅ (N) ₅ GAAC(N) ₅ CTC(N) ₅ (N) ₅	list	*548/543+580/575
32	PpuMI	RG ⁺ GTC ⁺ Y	list	330/333
33	PshAI	GACNNNNNNNN	list	*840
34	PspXI	VC ⁺ TGAGG	list	*999/1003
35	PvuI	CGAT ⁺ CG	list	*1473/1471
36	PvuII	CAGCTG	list	1132

TgABC-F

#	Enzyme	Specificity	Sites & flanks	Cut positions (blunt + 5' ext. + 3' ext.)
1	AvrII	C ⁺ CTAG ⁺ G	list	1146/1150
2	BaeI	A ⁺ (N) ₅ (N) ₅ ACNNNNNNNNNNNNNNNN	list	2196/2191+2229/2224
3	BamHI	G ⁺ GATC ⁺ C	list	111/115
4	BanI	G ⁺ GATC ⁺ C	list	*1354/1358
5	BciVI	GTATCC(N) ₅ NN ⁺	list	2197/2196
6	BfaI	C ⁺ TAG ⁺ G	list	1147/1149
7	BfuAI	ACCTGNNNNNNNNNN	list	371/375
8	BnuI	ACTGGNNNNNNNN	list	2299/2298
9	Bpu10I	CC ⁺ TAA ⁺ GC	list	952/955
10	BsaXI	A ⁺ NN ⁺ (N) ₅ AC(N) ₅ CTCC(N) ₅ NN ⁺	list	1765/1762+1795/1792
11	BsgI	GTGCGAG(N) ₅ NN ⁺	list	360/358
12	BsiWI	C ⁺ GTAC ⁺ G	list	*1238/1242
13	BspDI	AT ⁺ CGAT	list	*1504/1506
14	BspHI	T ⁺ CATGA	list	1538/1542
15	BspMI	ACCTGNNNNNNNNNN	list	371/375
16	BsrGI	T ⁺ GTAC ⁺ A	list	1103/1107
17	BstBI	TT ⁺ CGAA	list	*763/765
18	BstEII	G ⁺ GTAC ⁺ C	list	906/911
19	Bsu36I	CC ⁺ TAA ⁺ GG	list	686/689
20	BtgZI	GGGATG(N) ₅ NN ⁺	list	*1756/1760
21	Clal	AT ⁺ CGAT	list	*1504/1506
22	DnaII	CACNNNNNNNN	list	1398/1392
23	DrdI	GACNNNNNNNN	list	*318/316
24	EcoNI	CCTNN ⁺ NNNAGG	list	1303/1304
25	EcoO109I	RG ⁺ GTC ⁺ Y	list	2249/2252
26	EcoRI	G ⁺ AATTC ⁺	list	539/543
27	HaeII	R ⁺ AGCG ⁺ Y	list	*976/972
28	MseI	T ⁺ TAA ⁺ A	list	2317/2319
29	MslI	CAYNNNNNNNN	list	1741
30	NmeAIII	GCCGAG(N) ₅ NN ⁺	list	266/264
31	NruI	TCCGCA	list	*1231
32	NsiI	A ⁺ ATGCA ⁺ T	list	1511/1507
33	PaeR7I	C ⁺ TGAG ⁺ G	list	*1245/1249
34	PmlI	CACGTC	list	*2093
35	PpuMI	RG ⁺ GTC ⁺ Y	list	2249/2252
36	PshAI	GACNNNNNNNN	list	1261

Appendix III

Diagram of pESC-Ura vector



pESC-Ura Multiple Cloning Site 1 Region
(Sequence shown 2180-2263, bottom strand)

SphI SpeI CspI SphII PstI
 GAA TTC AAC CCT CAC TAA AGG GCG GCC GCA CTA GTA TCG ATG GAT TAC AAG GAT GAC GAC GAT AAG ATC TGA GCTCTTAATTA
 M D Y K D D D K I stop
 FLAG group

pESC-Ura Multiple Cloning Site 2 Region
(Sequence shown 2936-3033, top strand)

SphI SpeI SphII PstI
 G GAT CCG TAA TAC GAC TCA TAG GGC CCG GGC GTC GAC...
 stop
 ...ATG GAA CAG AAG TTG ATT TCC GAA GAA GAC CTC GAG TAA GCTTGATACCGCGGTAGC
 M E Q K L I S E E D I E
 acci group

Feature	Nucleotide Position
Yeast <i>URA3</i> selection marker ORF	417-1217
fl origin of ss-DNA replication	1483-1789
Yeast <i>ADHI</i> terminator	1855-2049
Multiple cloning site 1	2180-2263
FLAG tag	2198-2224
Yeast <i>GALI1</i> / <i>GALI10</i> divergent promoters	2268-2934
Multiple cloning site 2	2936-3033
c-myc tag	2976-3011
Yeast <i>CYC1</i> terminator	3038-3227
pUC origin of replication	3414-4081
Ampicillin resistance (<i>bla</i>) ORF	4232-5089
2μ yeast origin of replication	5223-6578

Above Figure is pESC-Ura vector. The complete sequence and list of restriction sites are available at www.stratagene.com. Note that the sequence shown for MCS1 corresponds to the bottom strand sequence (the reverse-complement of the sequence available for this vector at www.stratagene.com).

Appendix IV

TgABC-B- pESC-Ura sequencing with GAL10-Fwd (Initiation Methionine is highlighted yellow)

AAGAATTTTTGAAAATTCGAATTC AACCTCACTAAAGGGCGGCCGCATGCCCTCGCATCCTAATTTGGAGAGTACTAC
 ACCGCCTGTCCACGACCACAGTGGTGGGCAGCCAACGGAGTCCTTAACCTCAATTTGTCAGAAGAAGATTGCAGACTAT
 GCGGTACCGGTGGAGAATTACTGGTCAACTGACCGTCTTTCGCCAGTGGCTCTTGACAGAGAGACCACGACTGTTTCAG
 AGCCACGCCCCATCCCCCTTGTCTATGCACTGTCTACCATAGGTAGCCGGAGTCCCGCGGCTGAGGTCAGAACTCCTC
 AGCTTCCAGATGTGGAGGTGCAAGTCCACCTTCGCCCTGAAAACGGTGCTGCACGAAGTGGGGTTGTTTCAGGTGCTCACA
 CCGCCTCGCGTTGTCACTCTTCATAAGGAAGCCGATGATTCTGTACAGATGAAAGAGACCGAACAAGCGAAGCGTGCT
 GCAAGGCTACAAGTGTTTCATACGTTTCACAAACCTCACATTCACCTCCTCCGCGAGGCCTTGACGCCAATCCGTGAAAG
 AATGGGCTAAAACGGCTTCCTGTACACAGTCGTCACGACAGGCAATTGCTGGTTCCGATTCTGTTTCGGGACCGTGTTTC
 AAAGGCGCCTCCTCCGTCCGCATATGCTCGCCCCGACAAATTCTTTGGGCTGAGCGGCTACTTTGCCCTGGCGAA
 ATTTGGGGAATCCTCGGTCCTTCTGGAGCAGGCAAAAGCA

MPSHPNLESTPPVHSGGQPTESLSICQKKIADYAVPVENYWSTDRLSPVALDRETTTVSEPRIPSCYALSTIGSRSPAEEVRN
 SSASRCGGASPPSPENGAARSGVVQLTPRVVTLHKEADSVQMKETEQAARLQVFIRFTNLFTPPRGLARQSVKEWA
 KTASCTQSSRQAIAGSDSVSGPCSKAPPSAYARPRQILFGLSGYFAPGEIVGILGPSGAGKS

TgABC-B-pESC-Ura sequencing with GAL10-Rev (Reverse complement) (Termination codon is highlighted yellow)

ACTTCACTACTTCTTACTACAGCTTCGGACCTGCCTACATGGCATTACATCTTTTCCAGCTGAGCGCGCCGTTATCTCC
 AGAGAAAGAAGCAGTAAGGCATATAAAGTGTCATGTTATCTTCTCGCCAAAACCATGATAGACCTCGTCGTCCAGATCT
 TTACTCCGTCAGTGTGGCTGGCAATCGTATATCCGCTTGTGGATTGCCCTCAGACCTTGGCGTGTTTATGGCTTTTGG
 GCTCAGCTGGTACTGCTTGTCTGCATAGCTCAAGCCATAGGTCAACTGATTGCTGCGATCGTTGTGGACGATGCGCGCC
 TAGGGGGTTTGTCTGTGTCAGTCATTCTGATTTCATCTCCATCAGTAGTGGCTTCTATGTTTCAGCAACAGAGATTAGGC
 CCATGGATATCTTGGTTTCGATGGTTGTCTTCCAGAACTACGCGGTGACAACTTCGTAATAGTTACAGTGGGATCGTC
 TTCGACACTCTCGTGCTCGGAGTTCTCTTCGTTCCCAACTTGCCAGGGCAGCCGATCACAGCAGAGATGATTACCCGC
 AGGTTCAACAACAGCACTTTCGCCTTTATCCAATATCACCTTAATGATTTGTACATGGCTAGCCATCAAACTAATGTGCTA
 TGGAGTCTCAAGAAATCTCTTAACTGAAGTCCGAGCGGCCGCACTAGTATCGATGGATTACAAGGATGACGACGA
 TAAGATCTGAGCTCTTAATTAACAATCTCGCCAGA

FTTSYYSFPGPAYMAFTSFPAERAVISRERSSKAYKVS CYLLAKTMDLVVQIFTPLWLAIVYPLVGLPSDLGVFMAFWAQLVL
 LVCIAQAIGQLIAAIVVDDARLGGLLLSVLISISSGFYVQQRLGPWISWFRWLSFQNYAVTNFVIVTVGSSSTLSCSEFSF
 PTCPGQPITAEMITRRFTTALSPLSNITLMICTWLAIKLMCYGVLLKSLKLS

Appendix V

Buffer and Media:

E. coli competent cell buffers

TFB-I Buffer	Ingredients	TFB-II Buffer	Ingredients
CaCl ₂	10mM	CaCl ₂	75mM
Glycerol	15%	Glycerol	15%
KOAc, PH 5.8	30mM	MOPS, PH 5.8	10mM
MnCl ₂ ·4H ₂ O	50mM	RbCl Autoclave	100mM
RbCl Autoclave	100mM	Store 4 ° C	
Store 4 ° C			

E. coli transformation media

SOB Media	Ingredients
NaCl	500mg
KCl	186mg
Tryptone	20g
Yeast Extract	5g
ddH ₂ O	Ad 1 L
Autoclave	

Add sterile MgCl₂ solution to final concentration of 10mM.

E. coli culture

LB Media	Ingredients	LB Plates	Ingredients
NaCl	10g	NaCl	10g
Tryptone	10g	Tryptone	10g
Yeast Extract	5g	Yeast Extract	5g
ddH ₂ O	Ad 1 L	Agar	15g
Autoclave		ddH ₂ O	Ad 1 L
		Autoclave	

*LB:Laria Bartani.100ug/ml Ampicillin) was added in a media cooled to 55 °C.

S. cerevisiae competent cell buffers

10x TFB-I Buffer	Ingredients	10x LiAc Buffer	Ingredients
Tris-HCl	10mM	Lithium acetate	1 M
EDTA	100mM		
Autoclave			

50%polyethylene glycol (PEG3350) solution: Filter sterilize

LiAc-TE buffer: 1 part 10x LiAc, 1 part 10x TE buffer, 8 part ddH₂O, Autoclave.

(PEG3350)- LiAc-TE buffer: 1 part 10x LiAc, 1 part 10x TE buffer, 8 part 50%PEG3350, Prepare fresh.

S. cerevisiae transformation media: Dimethylsulfoxid (DMSO)

S. cerevisiae culture

YPD Media	Ingredients
Dextrose	20g
Peptone	20g
Yeast Extract	10g
ddH ₂ O	Ad 1 L
Autoclave	

YPD: Yeast peptone dextrose

SD Media	Ingredients	SD Plates	Ingredients
Yeast nitrogen base	0.85g	Yeast nitrogen base	0.85g
Ammonium sulphate	2.50g	Ammonium sulphate	2.50g
ddH ₂ O		Agar	10g
Autoclave	Ad 425 ml	ddH ₂ O	Ad 425 ml
		Autoclave	

*SD: Synthetic dextrose minimal media (SD drop out media)

Add 1x amino acid and 2% appropriate sugar.

10x amino acid mix

Amino Acid	Concentration (mg/liter)	Amino Acid	Concentration (mg/liter)
Adenine sulfate	40	L-phenylalanine	50
L-arginine (HCl)	20	L-serine	375
L-aspartic acid	100	L-threonine	200
L-glutamic acid	100	L-tryptophan *	40
L-histidine *	20	L-tyrosine	30
L-leucine *	60	L-valine	150
L-lysine	30	Uracil *	20
L-methionine	20		

*The omission of histidine, leucine, tryptophan, and uracil allows selection of plasmids harboring the cDNA of interest.

40% sugar stock (glucose) in ddH₂O: Filter sterilizes and Store at 4 ° C

Agarose gel electrophoresis

50x TAE buffer Ph 8.0	Ingredients
Acetic acid	57.1ml
EDTA	0.5M
Tris-HCl	242g
ddH ₂ O	Ad 1L
Autoclave	